

VASCULITIS IN BORDER DISEASE OF SHEEP

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GENERAL CONTENTS

VOLUME ONE

| | Page |
|--|------|
| SUMMARY | i |
| GENERAL INTRODUCTION | iv |
| CHAPTER I HISTORICAL REVIEW OF THE LITERATURE ON BORDER DISEASE AND REASONS FOR THE STUDY | 1 |
| CHAPTER II CLASSIFICATION OF BLOOD VESSELS | 16 |
| CHAPTER III RE-APPRAISAL OF THE VASCULITIS IN BORDER DISEASE LAMB TISSUES ON FILE | 24 |
| CHAPTER IV THE EFFECT OF FOETAL AGE AT INOCULATION | 36 |
| CHAPTER V DETAILED MORPHOLOGICAL STUDY OF THE VASCULITIS: LIGHT- AND ELECTRON-MICROSCOPY AND LIPID HISTO- CHEMISTRY | 54 |
| CHAPTER VI IMMUNOLOGICAL AND HISTOCHEMICAL STUDIES OF THE VASCULITIS | 86 |
| CHAPTER VII THE MATERNAL LESIONS OF VASCULITIS | 107 |
| CHAPTER VIII GENERAL DISCUSSION | 113 |
| ACKNOWLEDGMENTS | 124 |
| BIBLIOGRAPHY | 126 |

VOLUME TWO

TABLES

ILLUSTRATIONS

APPENDICES

S U M M A R Y

Aspects of pathogenesis and pathology of vascular lesions in Border disease of sheep have been investigated.

Morphologically the lesion is recognised by the development of microscopical lymphocytic-macrophagic nodules, located mainly in the adventitia of small arteries and arterioles of certain tissues especially CNS. In addition there were minor degenerative changes, characterised by the fragmentation of the nuclei of infiltrating and smooth muscle cells in the outer layers. Light- and electron- microscopically and histochemically virtually all the cells of the infiltrate are typical- and atypical- lymphocytes and macrophages. Evidence is provided that the lymphocytes originate from circulation and after traversing the arteriolar wall lodge in the adventitia. Occasional cells identified morphologically and histochemically as neutrophils could not be confirmed electron-microscopically. Plasma cells and their precursors were entirely absent in the infiltrate. Evidence of phagocytic activity in macrophages was obtained from the EM findings of secondary lysosomes, phagocytosed and free necrotic cell debris, residual bodies and an unidentified structure. However, EM did not demonstrate cells rich in intracytoplasmic RER or polyribosomes i.e. plasma cells capable of producing free antibodies. Nor did EM demonstrate localisation of circulating antigen-antibody complexes, and no viral particles were evident at the magnification used. Furthermore, there was no evidence of fragmentation or duplication of the elastic laminae, proliferation and transformation of smooth muscle cells, fibrinoid degeneration or fibrin deposition. The results with the FITC conjugated rabbit anti-sheep fibrin serum eliminated the slightest possibility there being minute fibrin deposits in affected vessels. The lesion appeared similar in all of the affected blood vessels and no healing was observed.

Histochemical techniques for lipid revealed droplets which according to their staining /

staining characteristics and extraction results are considered to be hydrophilic lipids probably cholesterol, cholesterol esters and triglycerides. These were present in and between the cytoplasm of infiltrated cells in the adventitia and occasionally were observed in the media of affected blood vessels. On the basis of their location, and the type of blood vessel affected, it is deduced that they are possibly due to minor degenerative changes in the media.

Study of the conditions under which the vasculitis was observed, revealed that the lesion is present only in infected animals and is therefore one of the manifestation of Border disease agent per se. However, vasculitis in both progeny and ewes is dependent upon gestational age at infection - \geq 82 days for progeny and $<$ 70 days for mothers. The optimum time to produce vasculitis, in terms of gestation is 110 days, when in two consecutive experiments 83% (10/12) and 100% (8/8) take was obtained in the related sub-groups.

A breed difference was also noticed during three consecutive experiments using Cheviot x Dorset-Horn (C x DH) and pure Dorset-Horn (DH) lambs, where C x DH lambs produced the lesion at earlier gestational ages, i.e. at 90 days gestation a period where DH failed to show the lesion.

Observations on a few goats revealed that the caruncular lesion can also occur in species other than sheep.

The earliest time post-inoculation that the ^{clearly developed} lesion was recognised was 21 days in progeny and 17 days in ewes.

It was found that the predilection site of the lesion is brain in progeny, whilst in the dam the lesion is only present in the caruncles. However, in progeny, vasculitis was also found in brain, spinal cord, epididymis, lung, mammary gland, sciatic nerve, heart, adipose tissue of kidney and lymph nodes, kidney, spleen, lymph node and skin in descending order of frequency.

Consideration of the relationship of the Border disease induced vasculitis to age at infection and survival time led to the hypothesis of an immune response associated /

associated with localisation of antigen on the blood vessel wall. In view of absence of demonstrable antibodies this immune response seemed likely to be either type III or type IV allergic reaction of the classification of Coombs and Gell (1968). With the direct and indirect immuno-fluorescent techniques, using conjugated and unconjugated hyperimmune serum against Border disease and rabbit anti-sheep IgG the localisation of antigen was demonstrated on the blood vessel wall. In vivo macrophage disappearance test applied to lambs and guinea-pigs sensitised with Border disease agent, showed 18.9% and 37.6% drop in the number of macrophages in the peritoneal exudate, compared with controls. These results support the immune hypothesis and favour a type IV allergic reaction.

It was found that the lesion was confined to arterioles and small distributing arteries. Infiltration of cells to the adventitia increased the thickness of this coat, resulting in an overall increase of wall thickness and consequently increased arteriolar $\frac{W}{L}$ ratio i.e. 1: 0.7.

The lesion was studied by montage of tracings of serial, H & E stained paraffin sections and found to consist of microscopic nodular swellings in the adventitia along the course of the affected vessels. These nodules are 60-282 μ in length and show a unilateral or bilateral segmental distribution with intervals of from 48-82 μ .

Comparison of Border disease induced vasculitis with other vascular conditions of mammals suggests that though there are some similarities to both periarteritis nodosa and the vascular changes of serum sickness these are so minor that the Border disease associated vasculitis may be considered a unique and separate entity.

GENERAL INTRODUCTION

Border disease is a transmissible, probably viral, disease of sheep. Pathognomonic microscopic findings in CNS are hypomyelinogenesis, hypergliosis and interfascicular lipid, and in the skin abnormal medullation of primary hair follicles. In the ewe, clinical manifestations of the disease are barrenness and abortion and a necrotising placentitis has been reported.

While much work in this field has been with infections at gestational ages earlier than 60-70 days, at which maximum production of characteristic Border disease is achieved, less attention has been paid to infections at the later stages of pregnancy and the investigation of the resultant pathological changes.

In a series of experiments on Border disease, conducted during 1967-1971 at Moredun Institute, a vasculitis was observed in lambs of ewes infected at 90 days gestation. The present work is directed toward the characterisation and pathogenesis of this vasculitis.

CHAPTER I

HISTORICAL REVIEW OF THE LITERATURE ON BORDER DISEASE
AND REASONS FOR THE PRESENT STUDY

CONTENTS

| | Page |
|--|------|
| A. Geographical distribution of the disease and related conditions | 2 |
| B. Characteristics of the disease in the field | 2 |
| C. Aetiology | 3 |
| D. Clinical findings | 6 |
| E. Post-mortem and histological findings | 9 |
| F. Immunology | 14 |
| G. Protocols for the diagnosis of Border disease: | 14 |
| 1. Progeny | 15 |
| 2. Ewes | 15 |

CHAPTER I

HISTORICAL REVIEW OF THE LITERATURE ON BORDER DISEASE AND REASONS FOR THE PRESENT STUDY

A. Geographical distribution of the disease and related conditions

The first report of Border disease was by Hughes et al, (1959) describing the occurrence of a congenital condition of lambs characterised by tremor, hairiness of the birth coat and low viability. In some affected lambs there was a deficiency of myelin in the CNS. In the same year under the title of "Hypomyelinogenesis congenita" Markson et al, (1959) recorded the occurrence in Yorkshire, the Isle of Wight and Montgomery of a disease which was similar in most respects though fleece abnormalities were not always present. Within the United Kingdom, Border disease has also been reported in Cumberland (Barr, 1964) and Scotland (Barlow, 1970b).

Across the world reports of similar conditions have come from New Zealand (Hartley and Kater, 1962; Manktelow et al, 1969), Ireland (Hamilton and Donnelly, 1970), California (Bell, 1967; Osburn et al, 1972), Canada (Darcel et al, 1961), Switzerland (Frankhauser, cited by Barlow, 1969) and Australia (Acland et al, 1972).

B. Characteristics of the disease in the field

In the earliest reports the disease appeared to be restricted to a few breeds - the Kerry Hill and Clun Forest, Leicester x Oxford, (Radnor x Welsh Mountain) x (Radnor x Suffolk). Since then an increasing number of pure breeds and crosses have been implicated totalling some 36-37 types and occurring under a variety of managerial conditions.

The disease is endemic in the Border counties of England and Wales and in flocks in which it persists it is encountered chiefly in the offspring of ewe hogs (Shaw et al, 1969; Shaw, 1971). However, ewes of all ages and parity are susceptible to the disease (Barlow, 1970b). In affected lambs there /

there is no evidence of sex predisposition nor apparently is there any effect of litter size; in the case of twins one or both may be affected though to varying degrees (Shaw, 1971).

Morbidity due to Border disease appears to be lower in flocks where the disease is endemic reaching 28-35% in lambs from ewe hoggs but only 0.3 - 1.7% in those from older ewes (Hughes et al. 1959). This would give an overall incidence in the flock of about 6% which accords well with the 1-6% annual incidence suggested by Osburn et al. (1972) for Californian farms. In new outbreaks, however, morbidity ranges from 13-50%.

Mortality is more difficult to assess. Some authors describe high mortality rates (Hughes et al. 1959) and others (Barr, 1964) considered mortality to be low. However, it is not clear whether figures are based merely on losses of live born lambs up to weaning or whether losses due to abortion, still-birth and apparent barrenness have also been included in the assessment.

C. Aetiology

In early reports a genetic aetiology of Border disease was favoured. This was based upon the apparent restriction of the disease to two breeds, the Clun and Kerry Hill, in a circumscribed geographical region, and also because the fleece changes resembled those described by Dry (1955 a and b) for the N type Romney sheep. In the Romney there are apparently two major non-allelic mimic genes which, if present, produce a great increase in the number of hairy fibres in the birth coat. One of these genes is an incomplete dominant (N) the other a recessive (nr) and they are located on different chromosomes. The homozygous recessive and the heterozygous dominant both give hairy fleeces but there is no evidence that CNS lesions occur.

The /

The transmissible nature of Border disease was first suspected when affected lambs and a ram which had previously been mated with "Border disease ewes" were introduced to a farm in an area where Border disease had not previously occurred. Subsequently Border disease appeared in the offspring of some of the local ewes which had been run with or been served by the imported animals. Normal lambs were born to local ewes maintained out of contact (Dickinson and Barlow, 1967).

Successful experimental transmission of Border disease was first reported in 1967 from two laboratories (Dickinson and Barlow, 1967; Shaw et al, 1967), following the injection of pregnant ewes with suspensions of tissues from affected lambs and an aborted foetus. Dickinson and Barlow used a brain/spleen suspension, Shaw and co-workers brain, cord, liver, kidney and lymph node.

Successful transmission has been achieved following injection by the subcutaneous, intraperitoneal, intravenous, intratracheal and oral/conjunctival routes. Oral, contact, coitus and pervaginal routes which might be expected to mimic the natural route closely have not been shown to be effective experimentally (Gardiner and Barlow, 1972; Porter et al, 1972 a and b). On the other hand, it might be that the foetus is not susceptible at the time when the ewe comes in contact with the agent. The least artificial of the successful experimental routes have been the intratracheal and oral/conjunctival which suggest that the natural route may be through the agency of droplets. The most generally used and reliable experimental route of transmission is the subcutaneous/intraperitoneal injection (Gardiner and Barlow, 1972).

The timing of injection in relation to gestational age may have an important bearing upon the outcome and different groups of workers have used a wide range of gestational ages - 27-72 days (Barlow and Gardiner, 1969), 8-126 (Shaw et al, 1967), 5 - 50 (Manktelow et al, 1969). Shaw et al. concluded /

concluded that the foetus was susceptible at gestational ages between 8-52 days. Barlow and Gardiner obtained a fairly uniform take over the whole of their age range, but Manktelow et al. did not find clinical disease in lambs from ewes injected earlier than the sixteenth day of gestation. It is clear that successful transmission can occur between 16 and 72 days gestation and there is some evidence that it may be most effective at about 52 days gestation. It seems that defective lambs with fewer of the characteristics of Border disease may result from maternal injection at gestational ages over 80-90 days (Shaw et al, 1967; Gardiner and Barlow, 1972; Jackson et al, 1972).

Reports of transmission of Border disease to species other than sheep are few and concern only goats. Abortion in one out of three pregnant goats was the only abnormality noted by Gardiner and Barlow (1972). Huck (1973) also injected goats with affected lamb tissues and obtained abortion and some kids with ill-defined abnormalities. He passaged the kid tissues in goats and then successfully challenged sheep with a goat passaged inoculum, thus concluding that other species could maintain the infection.

Allusions to a viral aetiology were made by Shaw et al. (1969, Barlow (1970b) and Carter et al. (1972), but it was not until successful transmissions were carried out using Penicillin and Streptomycin treated cell-free extracts of affected lamb brain and spleen (Gardiner and Barlow, 1972) that there was any evidence for this suggestion. Using Barlow's (1972b) protocols for the disease in infected ewes Gardiner et al. (1972) further showed that the causal agent was destroyed by mild heat (60°C for 90 minutes) di-ethyl ether (18 hours at 2°C) and in serial filtration experiments (450 - 35 nm. a.p.d.) showed that it was withheld only by the 35 nm filter. Using Black's (1958) formula they deduced a particle size /

size of 27 nm for the agent causing placentitis in Border disease infected ewes. They concluded that the agent was a virus probably a small RNA virus according to Andrewes and Pereira's (1967) classification.

Further support for a viral aetiology came from the now well-confirmed observation (Acland et al, 1972) that mothers of affected lambs have high titre antibody to the BVD/MD complex of viruses though in preliminary studies (Plant et al, 1973) there appeared to be differences between the Border disease virus and that of the NADL strain of BVD virus.

D. Clinical findings

Descriptions are given by Hughes et al. (1959); Markson et al, (1959); Barr (1964) Barlow and Dickinson (1965); Davison and Oxberry (1966); Nott and Shaw (1967); Shaw et al, (1967 and 1969); Barlow and Gardiner (1969); Manktelow et al, (1969); Barlow (1970a and b); Lewis et al, (1970); Shaw (1970 and 1971); Barlow, (1972a and b); Carter et al, (1972); Gardiner and Barlow (1972); Hamilton and Donnelly, (1972); Porter et al, (1972a and b); Osburn et al, (1972); Huck, (1973) and Terlecki et al, (1973).

In ewes with the natural disease there is no evidence of any clinical upset prior to abortion, though an unconfirmed report (Shaw et al, 1967) has indicated that there may be a mild pyrexia (2.5°F) 6-11 days after experimental infection. Infection of pregnant ewe hoggs may retard their subsequent growth (Shaw, 1971). In the experimental disease abortion has been observed between 37-143 days gestation and may reach an incidence of 30% or more (Barlow and Gardiner, 1969). Foetal death most commonly occurs 30-40 days post-infection (p.i.) but the interval between death and expulsion of the foetus is very variable. Early abortion may go unnoticed and the ewe regarded as barren.

Foetuses may be aborted fresh, macerated or mummified. In addition to clinically affected full term lambs there may be live-born premature lambs or full term still-births. The mean gestation period of affected lambs /

lambs may be shorter than normal - 2.8 days in one experiment (Barlow and Gardiner, 1969) and frequently it has been stated that affected lambs are smaller than controls at birth, e.g. affected Dorset Horn lambs 2.51 ± 0.28 Kg body weight, 39.0 ± 1.61 Cms. poll to rump; compared with controls 3.85 ± 0.15 body weight, 45.9 ± 0.96 Cms. poll to rump (Shaw et al, 1969). In another experiment in the same breed of sheep smaller differences of the same type were found (Terlecki et al, 1973). Furthermore, Barlow et al. (1970) found that by 90 days gestation affected fetuses were significantly lighter but not significantly shorter than controls. Earlier than 90 days gestation no effect of the disease on body size was evident.

In most reports mention is made of the abnormal conformation of lambs affected with Border disease. Typically the lambs appear stunted with short bodies, domed heads, incompletely extended forelegs, displaced and disproportionately small orbits and fine limb bones. For several of these parameters differences between control and affected lambs have been quantified and found statistically significant (Terlecki et al, 1973).

Gardiner in 1967 examining unsuckled lamb sera demonstrated the presence of 7S γ globulin (IgG, immunoglobulin) in 77% of lambs with experimental Border disease and in 34% of field cases. Placental transfer of immunoglobulin does not normally occur in sheep (Barboriak et al. 1958) but foetal production of antibody (Ab) to a variety of antigens (Ag) has been demonstrated (Silverstein et al, 1963a & b). Gardiner concluded that this globulin was of foetal origin but subsequent work (Gardiner et al, 1973) has revealed that this immunoglobulin is not directed against the virus of Border disease. Furthermore, Patterson and Sweasey (1969) were unable to confirm Gardiner's findings, though Jackson et al. (1972) demonstrated both IgM and IgG in pre-colostral blood /

blood from one of their lambs. Thus examination of pre-colostral sera though non-specific may be an aid to clinical diagnosis.

The more specific clinical signs of Border disease are tremor and hairiness of the fleece both of which may be present to different degrees and do not appear to correlate with each other (Barlow and Gardiner, 1969).

Very severely affected lambs have a weak general appearance. The bleat is weak, tremulous and pitiful. They have difficulty in standing or may be wholly recumbent. The neurological signs are least obvious during sleep and most pronounced during locomotor activity. They consist of rhythmic tonic/clonic spasms with a great range of both amplitude and frequency and affect head and neck, body and limbs. When they are severe they may render the lamb incapable of suckling unaided or making purposive movements. Nystagmus may be present and microphthalmia has been reported. Less severely affected lambs may be able to follow their mothers and suckle. Appetite is usually good. The neurological signs tend to resolve with time and partial recovery may commence at about three to four weeks of age.

In the fleece the fundamental abnormality is an increase in the proportion of hair in the woolly birth coat (Figs. 1 and 2). The hairs are long and wavy and may be abnormally pigmented for the breed. Some stand out from the fleece like a halo. These integumentary signs are more readily seen in smooth coated breeds such as the Dorset Horn. Experimentally it has been shown that no fleece changes are present in lambs from ewes inoculated earlier than sixteen days gestation (Manktelow et al, 1969) or later than 118 days gestation (Jackson et al, 1972).

Hairiness and pigmentation are most prominent on the poll, back, shoulders and rump. Halo hairs are lost as the birth coat changes to a more mature fleece but there is a tendency for the pigmentation to persist throughout life (Shaw, 1971).

When confined, lambs affected with Border disease are very liable to /

to traumatic injuries due to treading by adults. Fractures, lacerated tails and deaths from fatal internal haemorrhages are not uncommon (Barlow, 1970b). Intercurrent diseases such as pneumonia and helminthiasis are very common causes of death and Barr (1964) has considered that they do not respond well to antihelmintics. Thus general constitutional weakness leading to traumatic injury and intercurrent disease results in few affected lambs surviving to weaning.

E. Post-mortem and histological findings

Ewes:

There are no reports concerning post-mortem examinations of ewes in naturally occurring Border disease but serial examinations following experimental infection have been carried out (Barlow et al, 1970; Barlow, 1972b). Gross changes appeared to be confined to the uterus from 20-40 days p.i. onwards and were associated with oedema of the foetus and its membranes. The arcade haematomata of the placentomes were very prominent and showed a peripheral rim of brown discolouration. On separating the cotyledon small whitish foci were sometimes seen in the basal third of the crypt zone of the caruncle. The caruncles also showed strip-like radial haemorrhages which sometimes became contiguous with the arcade haematomata. Enlargement of the pelvic lymph nodes was observed but this was not specific to the Border disease injected ewes.

Microscopically changes were first seen ten days p.i. The number of lesions per placenta varied according to gestational age at inoculation. However, the nature and progress of the lesion was similar for all the inoculation ages examined. The lesions consist of necrosis of placentomal septa and apparently start as mural necrosis of the maternal capillary walls. The necrosis spreads to the crypt lining which gives way and haemorrhage takes place. The sanguinous and necrotic cell debris ruptures the microvillous union between the trophoblast and the crypt lining. There is some /

some phagocytosis of this debris by macrophages. The vasculitis which progresses in septal capillaries is characterised by endothelial swelling, mural necrosis and luminal occlusion sometimes associated with lymphocyte containing thrombi. At about 25-30 days p.i., healing of the caruncular lesions occurs due to phagocytosis of the necrotic debris by macrophages, calcification and stromal proliferation. In a proportion of ewes, however, the placental lesions become diffuse resulting in foetal death and abortion.

Progeny:- In experimental Border disease, gross changes in the conceptus have been described from about 75 days gestation (> 25 days p.i.) (Barlow et al, 1970). The foetal membranes were often dark coloured and oedematous, fragile and sticky to the touch whilst the branches of the umbilical vessels were shrunken and opaque compared with controls. The foetal skin of 75-80 day fetuses often had a yellowish-brown discolouration. Slightly older fetuses (90 days) and their membranes were not infrequently distended by a sero-sanguinous or gelatinous oedema fluid. Such fetuses were usually moribund or dead.

In the new-born lambs affected with either the natural or experimental disease the gross post-mortem examination is often unremarkable (Markson et al, 1959; Hughes et al, 1959). However, Barr (1964) observed swelling of the brain in eight week old lambs and Terlecki et al. (1973) reported a dome-shaped distortion of the cerebellum which they attributed to the abnormal conformation of the skull. In some 20% of experimental cases gross softenings of the cerebral white matter have been observed which resembled those of congenital swayback (Barlow and Gardiner, 1969). Such lesions have not yet been reported in naturally occurring Border disease, however.

In /

In a proportion of natural cases the brain appeared small, the lateral ventricles dilated, the spinal cord narrower and much firmer than is normal. (Barlow and Dickinson, 1965).

References to extra-neural pathology in Border disease are few. Hypoplastic kidneys with residual foetal lobulation have been observed (Barlow and Gardiner, 1969). Emaciation, lymphadenopathy, gastro-intestinal and pulmonary disease have all been described from time to time by several writers but are probably attributable to mechanical difficulty in feeding and to secondary inter-current disease.

The most consistent lesions of Border disease are microscopic and are located in the CNS and skin. The severity of tremor and the degree of neuropathological changes correlate fairly well. In the most severe clinical cases the CNS lesions are diffuse and profound, whereas in milder cases they are less severe but still diffuse or they are patchy with a partially perivascular distribution (Barlow and Gardiner, 1969). Defective myelination or hypomyelinogenesis is the dominant feature of the pathology and is readily demonstrated with the LFB, Loyez, OTAN or other myelin stains (Figs. 3 and 4) (Markson et al, 1959; Hughes et al, 1959; Barlow and Dickinson, 1965). All writers agree that the lesion is an interference with the process of myelination rather than a destruction of formed myelin. In the experimental disease hypomyelinogenesis is detectable as early as 75 days gestation (Barlow et al, 1970). Furthermore, examination of newborn cases of Border disease by electron microscopy has shown numerous unmyelinated axons, promyelin fibres and abnormally thin myelin sheaths (Cancilla and Barlow, 1968). However, the electron microscope also revealed some slight evidence of demyelination namely splits at the intra-period line some of which developed into cavities containing granular glycogen-like material. In older animals occasional swelling and fragmentation /

fragmentation of sheaths occurred with invasion by macrophages at the region of the nodes of Ranvier.

Hypergliosis is the other prominent feature of the CNS in Border disease. The usually orderly rows of oligodendroglia have a haphazard distribution (Hamilton and Donnelly, 1972) and according to Barlow (1972a) were significantly more numerous in a standard field of a standard region of ventral spinal cord of Border disease affected lambs than in the same region of controls. However, Barlow's assessment was only a density measurement and may not reflect absolute numbers. About half this glial cell population are rod cells (Hamilton and Donnelly, 1972) whilst other cells especially in older cases have large vesicular, folded or bizarre shaped nuclei (Barlow and Dickinson, 1965) which Cancilla and Barlow (1968) tentatively suggested might be astrocytes in view of the frequency with which they observed astrocyte processes in their preparations.

The proliferation of glial fibres observed with the electron microscope may well account for the firmness of the tissue observed at post-mortem examination.

Associated with the interfascicular glia appropriate histochemical methods (OTAN, Sudan IV) have revealed accumulations of tiny rather granular-looking lipid droplets which were more numerous in younger cases of Border disease (Barlow and Dickinson, 1965; Barlow and Gardiner, 1969; Storey and Barlow, 1972). These workers considered these interfascicular lipid droplets to contain esterified and non-esterified cholesterol. However, Patterson et al (1971) were unable to confirm the presence of similar droplets in their own Border disease material.

Histological examination of the malacic foci encountered in the cerebral white matter of a proportion of cases indicated their essentially destructive nature; they contained large foamy macrophages with variable amounts of free fat and showed glial proliferation especially in the vicinity of blood vessels (Barlow and Gardiner, 1969).

Despite /

Despite the severity of myelin lesions in Border disease, pathological changes in the neurone have rarely been mentioned in the literature and appear to be infrequent. Occasional chromatolysis and degeneration of Purkinje cells and large motor neurones of the brain stem and ventral horns of the spinal cord have been encountered in affected lambs several weeks old (Barlow and Dickinson, 1965; Barlow and Gardiner, 1969). They might be specific changes possibly arising secondary to the glial scarring and infrequent Wallerian type degeneration of their axons observed with the electron microscope; alternatively they may be the result of exhaustion due to persistent tremor. In the latter event they might be expected to be more widespread and frequently encountered.

In a proportion of the experimental material produced by Barlow and co-workers a vasculitis in the meninges and superficial brain substance has been encountered in lambs born to ewes injected in mid and late gestation (Barlow, personal communication). In one group the frequency was 9 / 24, the lesion appearing as a heavy infiltration of the vessel wall with mononuclear cells. This review of the literature has failed to expose any report of a similar type of lesion.

Abnormalities of the fleece are fairly consistent features of Border disease. The main histological change is an increased diameter of the hair follicle resulting in an increased frequency of medullated wool fibres (Carter et al, 1972). Using a quantitative method it was found that 37% of wool fibres in affected Dorset Horn x Cheviot lambs 0 - 3 days old were medullated compared with only 3% in controls (Barlow, 1972a).

Histological examination of the hypoplastic kidneys sometimes encountered has revealed a narrow cortex with a proportion of immature glomeruli and abundant peri-pelvic mesenchyme sometimes infiltrated by focal accumulations of round cells (Barlow and Gardiner, 1969).

F. /

F. Immunology

The earlier mentioned observation (Shaw et al, 1967) that in the field ewes do not usually produce affected offspring again at subsequent lambings gives rise to the notion of resistance. Primary outbreaks of Border disease may be severe because there is no resistance in the flock, whereas in flocks where the disease is endemic only those animals being bred for the first time will (as a group) be susceptible. On this assumption vaccination was attempted by Shaw et al. (1969). They injected ewes with virulent inoculum 20 days before mating and challenged them 20 days after mating. This treatment reduced the incidence and severity of the clinico-pathological manifestations but it did not entirely prevent the disease (Shaw, 1971).

et al
Gardiner (1973) have been able to neutralise the Border disease agent by inactivating infective tissue suspensions with sera from previously infected ewes "hyperimmunised" with repeated doses of infected tissue suspensions with or without Freund's adjuvant.

F. Protocols for the diagnosis of Border disease

From the foregoing review, it would seem that diagnosis of Border disease in an individual may be difficult especially since many cases may appear to become clinically normal as they grow older. However, in a number of experiments conducted over several successive seasons the standard inoculum used at Moredun has been shown to be highly effective in the reproduction of Border disease in the offspring of susceptible ewes. Variation in the timing of injection may affect the pattern of the characteristic pathological changes. Nevertheless, experience is sufficient to be able to consider with high degree of confidence that injection will result in an infection rate of 90% or better.

The diagnostic criteria of Border disease may be listed as follows:

1. /

1. Progeny: In this class of animals the diagnostic criteria may be variable according to the gestational age at which infection/injection occurs. For the individuals arising from infections/injections taking place in the first two-thirds of pregnancy^{the} following criteria may be used.

a. Clinical:

i) Live lambs - tremor, hairy coat, low birth weight and reduced crown rump length and abnormal conformation.

ii) Aborted or still-born lambs - hairiness of the coat

b. Pathological:

i) Hypomyelinogenesis of the CNS, i.e. a diffuse or focal absence or deficiency of myelin relative to the stage of development

ii) Hypergliosis - an increased density of interfascicular glial cell nuclei

iii) Interfascicular lipid: the presence of aggregations of granular, myelin-like lipid in association with interfascicular glial nuclei

However, from infections arising in the last third of pregnancy, live-born lambs may or may not be clinical shakers but will not be hairy. The CNS lesions will tend to be milder and more restricted in distribution. There is some evidence to suggest that vasculitis may be a poorly understood and hitherto undescribed consequence of infection in this class of lambs.

2. Ewes: Injected at any age develop a placentitis and may abort. At injection earlier than about 90 days gestation, clinically affected lambs may result or the ewes may appear barren probably because early abortion was unrecognised.

CHAPTER II.

CLASSIFICATION OF BLOOD VESSELSCONTENTS

| | Page |
|---|--------|
| A <u>Arteries</u> | 17 |
| 1. Elastic arteries | 17 |
| 2. Muscular (distributing) arteries | 19 |
| 3. Arterioles | 20 |
| 4. Precapillary or terminal arterioles | 21 |
| 5. Metarterioles | 21 |
| B <u>Veins</u> | 22 |
| 1. Large veins | 22 |
| 2. Small and medium size veins | 22 |
| 3. Venules | 22 |
| 4. Prevenules | 23 |

CHAPTER II

CLASSIFICATION OF BLOOD VESSELS

CLASSES OF BLOOD VESSELS

Descriptions are derived from Ham (1969), Heath et al, (1968) and Trautmann and Fiebiger (1957),

Blood vessels are divided into arteries and veins, each of which form several classes. ^(Fig 5) Each class contains many sub-classes the distinctions between which are not clear-cut. Classification and the light - and electron - microscopic descriptions of these vessels can be found in any histological text-book.

A- ARTERIES- There are three kinds of arteries, namely elastic, muscular (distributing) and arterioles. Each is composed of three coats, i.e. intima, media and adventitia.

1- Elastic arteries: (Fig. 7) e.g. aorta, pulmonary artery, carotid artery and so on. The size of these arteries is variable according to the species and age. This class of arteries are engaged in maintenance of pressure during diastole. Usually they run near a companion vein and are not embedded in a connective tissue mass as are the smaller vessels.

The intima is usually one-sixth (Fig. 6) of total thickness of the arterial wall. It looks paler than the media under the microscope. In addition to endothelial cells, the constituent cells of this coat are undifferentiated muscle cells, monocytes, macrophages, fibroblasts and collagen fibres. The intima contains less elastic fibres than the media. This coat is circumscribed externally by the well marked internal elastic lamina of the artery.

The /

The media is the thickest coat of the three and consists chiefly of elastic laminae which are arranged concentrically. The number and thickness of the laminae varies with age. In between adjacent laminae lie differentiated muscle cells. The outermost lamina is termed the external elastic lamina. However, histologically this coat in the elastic arteries of ruminants, mainly aorta and pulmonary arteries differ from that of dog and human. Ruminants have a dense musculo-elastic structure. Smooth muscle cells form thick bundles arranged in a complex oblique manner. On transverse section these present as discrete muscular nodules scattered throughout the media mainly in the outer two-thirds of this coat. Muscle bundles are devoid of elastic tissue and collagen and are separated from each other by dense bands of elastic fibrils forming thick fasciculi. In the inner third of the media there is a concentration of shorter elastic laminae, many of which are oriented longitudinally. An exception is the llama in which elastic arteries more closely resemble those of non-ruminant series; there is quite a sharply defined sub-intimal layer of circularly oriented elastic fibrils, and the outermost part of the media is mainly muscular with sparse elastic tissue between the muscular nodules, located at the junction of media and adventitia (Heath et al., 1968).

The adventitia is a thin coat. It consists of irregularly arranged connective tissue bundles which contain both collagenous and elastic fibres. Vasa vasorum (capillaries of vessels) and lymphatics are present in this coat.

The /

The Vasa vasorum penetrate as far as outer part of T. media. Hence, the inner layer of the vessel is nourished from the lumen whilst the outer layers of this coat receive nutrients via vasa vasorum. The T. media is most remote from a supply of nutrients and thus is probably more susceptible to degenerative changes.

2- Muscular (distributing) arteries: Fig. 8, e.g. coronary arteries

As the name implies the media of these arteries is rich in muscle fibres, and the latter are under the control of nerve impulses, they serve for the regulation of blood pressure and flow in different parts of the body. Nervous impulses reach the media through the efferent non-myelinated sympathetic nerves and the axonal reflexes of myelinated fibres, both of which have a vasoconstrictor effect on muscle coat.

Intima: This coat in small (Fig. 8 B4) and ⁱⁿ large-muscular arteries (Fig. 8 B2) varies slightly. The main difference is in the disposition of endothelial cells. In small muscular arteries the endothelial cells lie directly on the internal elastic lamina, whereas in larger type, endothelial cells rest on structures called musculo-elastic cushions. These consist of poorly differentiated muscle fibres distributed longitudinally. Musculo-elastic cushions consist of two layers superficial and deep. The former contains more amorphous intercellular substance and few fibres, whereas the deep layer contains more fibres. Other cells such as macrophages and fibroblasts are distributed in both layers. The external border of the intima is the internal elastic lamina which sometimes is double without being of any pathological significance.

Media: /

Media: Is the thickest and consists chiefly of circumferentially disposed well-differentiated muscle cells held together by reticular, collagenous and delicate elastic fibres. The proportion of inter-cellular substance in relation to differentiated muscle varies with the size of artery, i.e. in small arteries a greater proportion of differentiated muscle is present and in larger ones more elastin.

Adventitia: The thickness of this coat varies in muscular arteries but usually it is one-half to two-thirds the thickness of the media (Fig. 6). The main constituent is connective tissue. The fibres are chiefly condensed elastic fibres but some collagen fibres are also present. The elastin is condensed to form the external elastic lamina which is applied to and is continuous with the outer border of the media. Vasa vasorum and lymphatics are also present.

3- Arterioles: Fig. 9 C1, 3, 5

Are usually arteries with an overall diameter of 100 μ or less, although some authors also classify considerably larger vessels as arterioles. The ratio of thickness of the wall of the vessel to the diameter of its lumen is 1:2 with a range of 1:1.7 to 1:2.7. These vessels have the same three coats as arteries.

Intima: Endothelial cells are attached directly or with a trace of intervening connective tissue to an internal elastic lamina. In smaller arterioles (Fig. 9 C3) the internal elastic lamina becomes very thin and in the smallest vessels it is absent altogether.

Media: Consists of concentrically arranged differentiated muscle cells. In the larger arterioles (Fig. 9 C1) the differentiated muscle cells are proportionately smaller. In the smallest (Fig. 9 C5) arterioles only one to two differentiated muscle cells constitute the media as seen in cross sections.

Adventitia /

Adventitia: May be as thick as the media and consists of a mixture of collagenous and elastic fibres. In the smaller arterioles (Fig. 9 C3) it is composed chiefly of collagen and is proportional in amount to the size of the vessel. In these very small arterioles elastic fibres are absent.

4- Precapillary or terminal arterioles: Fig. 9 C7

These are very small arterioles with a lumen not larger than the diameter of a red blood cell. The wall does not have any internal elastic lamina and consists only of endothelium applied to a layer of differentiated muscle cells and surrounded by a little connective tissue.

5 Metarterioles: These are seen on electron microscopic level. They are arterioles in which the differentiated muscle cells are replaced by cells called perivascular cells or pericytes. The distinction of these two types of cells is based on the intracytoplasmic organelles and cytoplasmic processes as is given in table I-4 which can be recognised at E.M.

B- VEINS - Figs. 8 B1, 3, 5

1- Large veins (Fig. 8 B1)

These vessels have in general three tunica as have arteries.

Intima: The endothelial cells rest on a rather thick sub-endothelial connective tissue. There is no distinct internal elastic lamina.

Media: Is much thinner than that of a companion artery. It consists mostly of collagenous and elastic fibres. Little differentiated muscle is present in the media.

Adventitia: Is the thickest of the three tunics and contains both collagenous and elastic fibres. In some veins the innermost layer of the adventitia shows bundles of longitudinally arranged differentiated muscle cells.

2- Small and medium size veins (Fig. 8, B3, 5 and 5')

As a whole they resemble the large veins. The difference is that endothelial cells rest directly on the internal elastic membrane.

The sub-endothelial connective tissue layer is thinner than in large veins. The innermost differentiated muscle cells of the media run longitudinally.

Cerebral and meningeal veins (Fig. 8 B5') have almost no muscle in their walls.

3- Venules (Figs. 9 C2, 4, 6)

These have a relatively larger diameter. The wall of the smallest ones consist of endothelial cells surrounded by a little collagenous connective tissue. Occasionally a single differentiated (smooth) muscle cell may be detected in the wall (Fig. 9 C6). Somewhat larger (medium-size) venules show the same constituents but the proportion of muscle cells is increased (Fig. 9 C4). However, in large /

large venules (Fig.9 C2) a proper muscle layer is detectable.

Distinction between this class of venules and small veins is a matter of the density of the muscle layer, collagenous and elastic fibres and the presence of longitudinally arranged muscle fibres in the innermost layer of tunica media of the small veins.

The muscle layer of these vessels is under nervous control. Therefore they play an important role in the regulation of the blood flow in the peripheral circulation.

4 - Prevenules (Fig. 9 C8)

These are vessels with a larger diameter than capillaries and consist of two to three endothelial cells supported by a thin layer of connective tissue.

CHAPTER III

RE-APPRAISAL OF THE VASCULITIS IN BORDER DISEASE LAMB TISSUES ON FILE

| <u>CONTENTS</u> | | | | | | | | | | Page |
|--|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| INTRODUCTION | ... | ... | ... | ... | ... | ... | ... | ... | ... | 25 |
| I. METHODS | .. | ... | ... | ... | ... | ... | ... | ... | ... | 25 |
| A- Selection of the Cases | ... | ... | ... | ... | ... | ... | ... | ... | ... | 25 |
| B- Staining | ... | ... | ... | ... | ... | ... | ... | ... | ... | 25 |
| II. RESULTS | ... | ... | ... | ... | ... | ... | ... | ... | ... | 27 |
| A- Description of the lesion and the result of special stainings | ... | ... | ... | ... | ... | ... | ... | ... | ... | 27 |
| B- Conditions under which the vasculitis was observed | ... | ... | ... | ... | ... | ... | ... | ... | ... | 29 |
| III. DISCUSSION | ... | ... | ... | ... | ... | ... | ... | ... | ... | 32 |
| IV. CONCLUSION | ... | ... | ... | ... | ... | ... | ... | ... | ... | 35 |

APPENDIX I = Vol. 2

INTRODUCTION

In the course of investigations conducted for the study of Border disease at the Moredun Institute between 1967-71 vasculitis had occasionally been observed. To study this phenomenon in detail the material obtained from these studies and available as paraffin embedded, haematoxylin and eosin (H & E) stained sections was used.

I. METHODS

A. Selection of the cases: Four cases diagnosed as positive for the vasculitis were examined in detail to gain familiarity with the lesion. These were amongst the experimental Border disease cases of 1969. Thereafter three natural cases of Border disease collected in 1970, and 222 of the total 486 experimental animals deriving from Border disease research between the years 1967 and 1971 were selected at random and examined. These consisted of 147 infected and 75 controls.

A summary of different classes of animals, from tissues of which the sections were available for study, is presented in table 2.

B. Staining

The tissues had been post-fixed in corrosive sublimate, mercuric pigment was removed by alcoholic iodine solution and hypo (5% thiosulphate solution) and the sections stained H and E. The following special staining techniques were used for sections positive for or suspected of having lesions of vasculitis.

1. Lendrum's carbol-chrometrope (C.C.) method to demonstrate eosinophil granulocytes.
2. Masson's trichrome stain (M.T.) for collagen and fibrous tissue.
3. Elastic tissue and its degenerative changes was demonstrated by Verhoff's haematoxylin elastic tissue stain. Occasionally Weigert's elastic method was also used.
- 4.

4. Lendrum's et al modified Martius Scarlet Blue (M.S.B.) technique was used to reveal fibrin and fibrin deposits. Occasionally Fuchsin-Miller technique was used for the same purpose.
 5. Mallory's phosphotungstic-acid-haematoxylin (PTAH) technique was used to demonstrate the thickening of tunica medialis and the striation of muscle fibres.
 6. Modified von Kossa's method for calcium salts deposits.
- All techniques (2 to 6) are from Drury et al. 1967.
7. Gordon and Sweet's technique (GS) to demonstrate distribution of reticulin fibres.
 8. Van Gieson's technique to reveal amyloid and hyalin degenerations.
 9. Benhold's Congo Red technique for the demonstration of amyloid.
 10. Jordan and Baker's methyl-green pyronin (M.G.P.) technique to reveal DNA and RNA activities in plasma cells.
- Techniques 7 to 10 are from Culling, 1963.
11. Nuclear stains: to reveal nuclear debris.
 - a. One per cent Safranin O.
 - b. One per cent Neutral Red.
 - c. One per cent Toluidine Blue.
 - d. Celestin Blue.

These were from Culling (1963) and Drury et al. (1967).

II. RESULTS

A. Description of the Lesion and the Result of Special Stains

The vasculitis observed in this part of the study could be classified under two headings, i.e. infiltrative (reactive) changes and degenerative changes. Cases diagnosed as positive for vasculitis showed either or both types of changes.

Infiltrative changes were the outstanding feature of the lesion and could be seen in almost every positive case. The infiltration was cellular (Fig. 10) and occurred mainly in the adventitial layer of the blood vessel, but occasionally it extended into the media or intima (Fig. 11) with reduced intensity. The constituent cells of the infiltrate were mostly mono-nuclears. (Figs. 10 and 12). These were small, medium- and large-lymphocytes, or macrophages with abundant cytoplasm and a large eccentric nucleus. Occasionally, amongst the mono-nuclear cells there were two to three cells with the characteristics of plasma cells, i.e. cells with a large eccentric nucleus with dense peripheral chromatin and a clear nuclear halo (Fig. 13). However, these cells did not respond to the MGP staining. They were presumed to be either immature plasma cells or macrophages. Neutrophils comprised only a small proportion of the population of infiltrative cells, but could be seen in all layers. At high magnification one to two eosinophil granulocytes were observed to be located in sub-endothelial layer.

Occasionally, when a longitudinal section of the blood vessel was /

was present in the preparation, it could be seen that the infiltrated cells made a bulge on the tunica adventitia (Figs. 14 and 15). This gave a nodular appearance to the blood vessel, and showed that the blood vessels are involved segmentally.

Degenerative changes consisted of vacuoles in the basement membrane, between endothelial cells and the sub-endothelial tissue. Endothelial cells were occasionally rounded and swollen. Oedema was evident - in the media and adventitia. The internal elastic membrane was intact in all cases. Elastic tissue stains (Verhoeff and Weigert) did not reveal any fragmentation or splitting of elastic membrane. Densely basophilic granular nuclear debris could be seen (Fig. 16 and 17 arrow) occasionally in the intima, media and adventitia amongst the cellular infiltrates. The distribution of reticulin fibres as demonstrated by the G.S. technique, appeared to be in normal range. Occasionally foci of dull, opaque eosinophilic material could be observed in the wall of blood vessels. Therefore, these were examined for the presence of fibrin, amyloid and hyalin precipitations and degenerations. Neither MSB nor Fuchsin Miller revealed any fibrin depositions, nor did van Gieson's or Benhold's Congo Red techniques demonstrate amyloid or hyalin degeneration.

Proliferative changes within arteries were present in the epididymis of one lamb. These were confined to the cells of the intima of small distributing arteries and projected into the lumen (Figs. 18 and 19). The media was minimally involved.

No /

No calcification, fibrosis, thickening or any sign of scar formation due to healing processes could be appreciated when the suspected sections were stained with von Kossa's modified technique or M.T. and PTAH methods.

B. Conditions under which the vasculitis was observed

The vasculitis was observed in 13 lambs born to the ewes inoculated with the Border disease agent.

In general, the animals received the inocula, infective or control, through the mother at the following gestational stages: 50 days, 54 ± 1 days, 60-75 days, 80-96 days and at 120 days. Two lambs were injected neonatally at 2-3 days of age.

In the Moredun experiments, infection with Border disease agent during the first 80 days of gestation in sheep has established the characteristic pathology of Border disease. However, Shaw et al. (1967) and Gardiner and Barlow (1972) have produced evidence that infection taking place during 78 to 120 days of pregnancy induce a pattern of lesions in which the characteristic histopathological changes of Border disease, i.e. wool dystrophy and myelin defects are either minimal or absent. Nevertheless, the agent produces lesions in the placenta which are ^{more} consistent in type and extent all the way through the pregnancy than are the CNS and integumentary changes (Barlow et al., unpublished data). Thus, in this work vasculitis is studied in relation to infection with Border disease agent, rather than in relation to the clinical disease per se.

Eighty-two days of gestation was the earliest age in which experimental inoculation of the Border disease agent into ewes gave rise /

rise to the lesion in the progeny. However, the lesion was observed in the progeny of ewes inoculated as late as 120 days and in the lambs receiving infective inoculum intracerebrally at 2 to 3 days of age.

Vasculitis was not observed in any of the large number of foetuses or lambs examined whose dams were injected at less than 82 days, nor in general in the tissues of mature sheep (c.f. Appendix 1). Hence the developing sheep is susceptible to the vasculitis of Border disease between 82 days of gestation and the early neonatal period.

Working within this frame of susceptible age at infection, it was noticed that the vasculitis was present in 13/26 lambs examined. Twenty-four were born to the ewes inoculated with infective inoculum, out of which eleven were positive for vasculitis. The other two were born to normal ewes but infected at two to three days of age.

This specific vasculitis was not observed in any of the lambs born to the control ewes, either those receiving normal brain suspension or uninjected.

The sites of vasculitis in lambs in descending order of frequency were brain (Figs. 14, 15 and 20), epididymis (Figs. 18 and 19), spinal cord (Figs. 11 and 21), adipose tissue from the vicinity of the kidney and lymph nodes, sciatic nerve (Fig. 12), spleen, lymph node, lung, and kidney. In all cases in which the visceral organs were involved, the lesion was present in the central nervous system (CNS) as well. However, in the majority of the cases only the C.N.S. was apparently involved. The nature and the number of tissues examined and the results of vasculitis in the susceptible group is presented in table 3.

According to the data available, the minimum survival time for the development of the morphologically recognisable vasculitis was 21 days. The lesion was also present in one lamb which was sampled 180 days post-inoculation. The details are provided in tables 4 and 5 of Appendix 1. However, in the fetuses or lambs infected earlier than 82 days, vasculitis was absent when examined between 5 to 300 days later.

Inocula, dose, dilution and routes of inoculations used for the production of Border disease varied with the experiments from which the lambs examined had been derived.

Details of dilutions of the inoculum, routes of inoculations, the effect of the sex, litter size and timing at inoculation and sampling are summarised in the tables 2 to 5 of Appendix 1. Though there may be trends emerging from the data, no effect of dilution, type of inoculum, route of inoculation, sex or the litter size could be clearly demonstrated. This should only be regarded as an interim conclusion, however, because of the small, irregular sample size in some groups. Data on the samples obtained from the progeny of ewes infected earlier than 82 days is provided in tables 6 to 10 of Appendix 1.

III. DISCUSSION

The presence of vasculitis in the progeny of the ewes and the lambs which had received the infective inoculum, provides evidence that the vasculitis may result from the action of Border disease agent, or some other parts of the inoculum. It might be argued that other substances in the inoculum, e.g. the foreign proteins and polysaccharides, with a high molecular weight, have initiated the lesion. If so, then the controls receiving the same type of proteins should have had the lesion also. This was not so and thus it seems that vasculitis is the result of the inoculation of Border disease agent per se.

As mentioned previously, characteristic lesions of Border disease i.e. wool dystrophy and myelin defects in the CNS of fetuses/or lambs, are produced as a result to the infection of the fetuses with Border disease agent at gestational ages earlier than 78 days. Vasculitis, however, was not observed in this class of lambs but only in those infected later in gestation in which the characteristic signs and lesions of Border disease are minimal or absent. It is thus clear that in the production of the vasculitis the timing of infection is important. Vasculitis did not occur in fetuses less than 82 days of gestation or in mature sheep. This suggests that to be susceptible to vasculitis the individual must have reached a certain stage of development yet still be immature. It must have reached a stage of development sufficient to allow it to mount a cellular response. This immediately raises the question that this vasculitis is related to developing foetal immune mechanisms. It is more difficult to speculate why the individual at a later, adult stage ceases to respond to /

to Border disease infection with vasculitis. Possibly the agent can only replicate significantly in immature tissue.

The idea that the vasculitis is immunological in nature gains some support from the work of Silverstein et al. (1963a), who showed that immunological competence in the foetal sheep begins at around 60 days gestation and with respect to some antigens it is ⁱⁿ an immunological "null" state until the 120 day of gestation.

In the progeny of the ewes inoculated at susceptible stages of pregnancy, the lesion is present 21-180 days post-inoculation. This suggests that for the development of the lesion a minimum survival time may be required. Furthermore, it seems that the lesion lasts for some considerable period of time.

The high incidence of vasculitis in the CNS and its relative infrequency in the other organs elicits the idea that CNS is a major target organ for vasculitis of Border disease. Hence, for the study of this lesion, the examination of the brain and spinal cord is essential. Why the incidence in the CNS is high is not clear. It may be due to any of several factors. One of the most likely reasons could be the localisation of high titre antigen (Ag) on the blood vessel walls of a tissue which is particularly susceptible to the effects of Border disease. Reaction of the antibody/s (Ab) against the localised antigen give rise to the lesion.

Another equally probable cause could be precipitation of circulating soluble Ag-Ab complexes. Both of these hypothesis can be investigated experimentally. Another relevant factor in the distribution of vasculitis could be the type of the blood vessel involved. This could be tested by classifying the types of affected blood vessels in the CNS and other organs.

Finally /

Finally, the occurrence could be the result of local Ab production, in the brain, against the precipitated antigen. If this be the case, then Ab-producing cells such as plasma cells should be present and capable of demonstration, particularly through electron microscopic (EM) studies. There is also the possibility that the lesion is the result of the local response of the CNS to the products of damaged brain. This is unlikely, since vasculitis has not been seen in cases injected at 54 days, when CNS damage is maximal. However, due to insufficiency of data, the frequency of the lesion in the organs other than those studied cannot be answered.

As the experiments had been devised originally for purposes other than the study of vasculitis, there are many gaps in the present data, which make conclusions difficult to reach. For instance, with respect to the effects of sex and the size of litter on the occurrence of vasculitis, the lesion tended to be more frequent in twins than singletons. Further experiments are required to answer these questions, e.g. the inoculation of Border disease agent into ewes at fixed intervals through gestation and the sampling of fetuses/or lambs at fixed intervals after inoculation for the examination of a wide range of tissues. It would then be possible to determine the exact stage of development at which susceptibility to vasculitis first occurs and the stage of development at which susceptibility ceases. It would also be possible to determine the exact "incubation period" for vasculitis and whether this incubation period or the distribution of vasculitis varies with age at inoculation. Once the conditions for the experimental induction of vasculitis /

vasculitis in Border disease are fully established, the lesion could be reproduced in a high frequency. Thereafter the other questions and aspects of the lesion as its incidence in the other organs; class of vessels affected most; EM, immunological and histochemical aspects could be studied easily. Work of this type would, however, be enormously expensive in terms of the time and experimental animals, and for its further development this study depends upon the integration of its objectives with those of other specific aspects of Border disease research.

IV. CONCLUSION

1. The vasculitis described in this part of the work is due to interaction of Border disease agent with foetal or newborn lamb tissues and occurs predominantly in CNS.
2. On the basis of present data, the youngest affected animal was 82 days of pre-natal life at infection of the dam and the oldest 2 days old.
3. An immunological basis for the lesion seems likely.

CHAPTER IV

THE EFFECT OF FOETAL AGE AT INOCULATIONCONTENTS

| | Page |
|---|------|
| INTRODUCTION | 37 |
| I. MATERIALS AND METHODS | 38 |
| A. Animals | 38 |
| B. Inocula and inoculations | 39 |
| C. Procedure of euthanasia, perfusion and post-mortem examination | 40 |
| D. Tissues sampled | 42 |
| E. Fixation, preservation, processing and staining | 43 |
| F. Assessment of the optimum CNS blocks for further studies | 44 |
| II. RESULTS | 45 |
| A. Age at inoculation | 45 |
| B. Survival time post-inoculation | 45 |
| C. Tissues | 45 |
| D. The effect of sex and litter size | 46 |
| E. Assessment of the optimum CNS blocks for further studies | 46 |
| F. Non-specific findings | 47 |
| III. DISCUSSION | 49 |
| IV. CONCLUSION | 53 |

APPENDIX II. Vol. 2

INTRODUCTION

This experiment was designed as a pathogenesis study of the placental, neurological and cutaneous lesions in Border disease to determine whether the IgG found in unsuckled Border disease lamb serum was foetal or maternal in origin. The material, however, also permitted the observations recorded in the previous chapter to be extended or confirmed.

I. MATERIALS AND METHODS

A - ANIMALS. 98 Cheviot ewes vaccinated against Louping Ill, with Brotherston and Boyce (1969 and 1970) new vaccine, were mated with either of two Dorset-Horn (D.H.) rams. Vaccination against Louping Ill was to check whether there is any leakage of maternal γ -globulin into foetal serum.

Positive matings were recorded by checking of mating marks on the back of the ewes. Failure to return to service was assumed to indicate conception.

Pregnant ewes were divided in three groups namely "A", "B", and "C". Group "A" was inoculated at the fiftieth day of gestation. They were destined to be sampled at five to ten days intervals thereafter through gestation. Groups "B" and "C" both were injected at ten days intervals through gestation from the fiftieth day of pregnancy. Group "B" was sampled at a standard time (fifteen days) post-inoculation. Group "C" were allowed to lamb. All the lambs were sampled three weeks post-natum, except the siblings of twins. These were kept for six months and then sampled. The older groups were larger and the sampling interval was greater. The bulk of evidence to the time of this experiment indicates that risk of abortion was highest around ninety days of gestation forty days post-inoculation.

Extensive experiments carried out on Border disease, over six to seven years, have shown that normal brain inoculations produce no lesions (Barlow, personal communication). Therefore, controls of Group "A" served as histological controls for Group "B" as well. Two un-inoculated ewes and their progeny served as controls for Group "C".

Four /

Four experimental and one control ewes, inoculated at the fiftieth day of gestation, and their progeny, which were killed fifteen days later served for both groups "A" and "B".

For the reasons outwith experiment, some ewes died. Some were barren and some aborted and thus provided no material for experimentation. From the remainder seventy-four fetuses and nineteen lambs as detailed in table 5 were available for study.

B. - INOCULA AND INOCULATIONS

All of the ewes, both infected and controls, received a 5 mls single dose of 5×10^{-3} dilution of appropriate inoculum subcutaneously and intraperitoneally (sc./ip.). One ml of it was inoculated i.p. and the rest s.c. In infected animals the inoculum consisted of saline suspension of homogenised unsuckled lamb brain pool, treated with Penicillin and Streptomycin (100 i.u./ml.) twenty to thirty minutes prior to inoculation. The brain was obtained from the third experimental passage of Border disease agent in sheep. For control animals a similar suspension was prepared from a normal brain in the same way as above.

C. PROCEDURES OF EUTHANASIA, PERFUSION AND POST-MORTEM EXAMINATION

At termination, ewes were anaesthetised i.v. through the jugular vein, using pentobarbital sodium (Nembutal, Abbott) at a dose of 1 ml/5 lbs. of body weight. The depth of anaesthesia was checked by respiration rate, pupillo-ocular and pedal reflexes. A laparotomy was performed and the uterus was exposed. Following incision of the uterine wall foetal blood was sampled from a branch of the umbilical artery using a 5 mls. syringe and a 19 gauge needle. Anticoagulant (0.1% heparin in saline = 160 u.i./ml.) were introduced to the foetus through a branch of the umbilical vein by means of a 22 gauge needle. As it is evident from the work of Romanes (1947) and Barlow (1969), the CNS is well developed in the sheep foetus by 105 days. To reduce pain and response to the manipulation, anaesthetic was given to the foetuses of 105 days and over, incorporated in the heparin saline injection. The volume and concentration was dependent on the age of the foetus as presented in table 6.

Placental membranes were divided, umbilical vessels double clamped and the cord lying between the clamps was severed and the foetus removed for further processing. Umbilical artery was cannulated and the foetus perfused with 1% glutaraldehyde in 0.1 M phosphate buffer solution (PBS) at a pH of 7.4. The perfusate was voided by controlled leak from an incision in the umbilical vein. The pressure and rate of perfusion were fixed to 110 to 125 cm. of water and 2 to 3 drops per second respectively. The volume of the perfusate and the gauge of the needle used was adjusted according to the age of the foetus as shown in table 7.

All foetuses and lambs were ^e weighed on a spring balance. The "crown rump" length (CRL) was taken as the length of the subject from the medial canthus of the eye to the root of the tail along the dorsum. Foetuses were weighed and measured prior to perfusion.

The lambs were killed by a section through the atlanto-occipital space. Post-mortem examination was carried out immediately on lambs, whereas the inspection of the fixed and perfused foetuses was postponed to a suitable time. Dead lambs were examined at the earliest time of notification. All subjects were examined by the same technique.

In every cadaver particular attention was paid to the circulatory system, examining closely the aorta, cerebral, coronary, carotid, coeliac, pancreatic, splenic, mesenteric, urinary, ovarian, uterine, iliac arteries and those of large and small omentum. Careful systematic examination was carried ^{out} on blood vessels, considering the adventitia (for petechiae, haematomas, granulations), diameter (aneurysms, atresias), straightness (any kind of kinkiness and irregularity). Then the blood vessels were opened with a pair of Mayo's scissors and the condition of vessel walls was examined. The media of the opened vessel was tested for variations in thickness. The intima was checked for smoothness (thrombosis, atherosclerosis, ulceration and hypertrophies). Particular attention was paid to the infarctions, ischemic changes and petechiae in the systemic organs, in all of which an underlying arteritis might be involved.

D. TISSUES SAMPLED

The range of tissues sampled from the foetuses and lambs was based partly on the results of Chapter III, with the addition of tissues not available in that study, and partly on the distribution of vasculitis in conditions reported in the literature. They covered almost every organ and were:

Skin, lymph nodes (prescapular, mediastinal, precrural and iliac), brain (at the levels shown in diagrams of Fig. 22, i.e. B1, B2, B3, B4a, B4b, B5, B6 and B7), spinal cord at C2, C7, T8 and L3, pituitary, eye (left), parotid gland, thyroids, thymus, aorta (anterior and posterior, heart (transverse block of the ventricles near the base of the heart across the descending branch of the coronary vessels), lung (left apical lobe), liver, gall-bladder, spleen, pancreas, rumen, reticulum, omasum, abomasum, duodenum, ileum, caecum, rectum, kidney, urinary bladder, testis, epididymis, prostate, uterus, and ovaries and mammary gland.

E. FIXATION, PRESERVATION, PROCESSING AND STAINING

Sampled tissues were utilised only for the light microscope (LM) study. Tissues either fresh or perfused were immersed, fixed and preserved in formol-calcium chloride solution (Baker, 1944) at room temperature. Specimens were also taken for frozen sections to perform histochemical techniques.

Blocks of Baker's fixed tissues were post-fixed in two changes of saturated mercuric chloride of 24 hours each. They were then washed in water, dehydrated in graded alcohols, and processed to paraffin blocks.

Paraffin sections were cut on rotary or rocking microtomes at 6 μ thickness for ordinary histological techniques, and 12 μ for Luxol-Fast Blue. Frozen sections were cut on Frigisto^{*} Thermo-electric stage^{*} microtome at 12 μ thickness for OTAN (osmium tetroxide- α -naphthylamine).

Mercuric pigment was removed as described in Chapter III. Staining techniques applied to the sections, in addition to H & E, were as in the previous Chapter unless otherwise stated.

1. Luxol Fast Blue (LFB) - to examine the degree of myelination.
2. Sudan IV - to demonstrate lipids in the arterial wall.
3. Gram's technique = to reveal bacterial organisms in appropriate cases.
4. Perles' Prussian Blue Reaction (PBR) = for haemosiderin.
5. Osmium-tetroxide- α -naphthyl-amine (OTAN) = for myelin and products of myelin degeneration (Adam's, 1959).

Techniques 1 and 2 were from Culling (1963) and methods 3 and 4 were from Drury et al. (1967).

* De la Rue Frigisto^{*} Ltd.

F. ASSESSMENT OF THE OPTIMUM CNS BLOCK FOR FURTHER STUDIES

The blocks of brain used for examination are shown in Fig. 22. In addition four levels of spinal cord were examined as detailed in the sub-heading "D" of this Chapter. Affected blood vessels were recorded for each case in relation to their anatomical location. Blocks showing the highest incidence of vasculitis in both this experiment and the material of Chapter III would be chosen as representative blocks for sampling in further studies. The other objectives of this technique were to see whether:

- a - If a specific blood vessel is affected are its branches also affected?
- b - Is the lesion present bilaterally in blood vessels with anatomically symmetrical distribution?

II. RESULTS

A - Age at inoculation

One foetus from group "B" and four lambs from Group "C" developed vasculitis. All five animals were in the previously determined age range of susceptibility at inoculation. The distribution of the number of animals in relation to the age at inoculation is summarised in table 8. According to the results of this experiment the "susceptible" age ranged between 90 to 130 days of foetal life. None of the controls or the animals from the ewes infected at earlier than 90 days were affected.

B - Survival time post-inoculation

The details of the survival time after inoculation and the number of animals examined from each age inoculation in relation to vasculitis is summarised in table 9. The shortest post-inoculation time at sampling at which vasculitis was present was 15 days, but this was in only one case out of a possible 14 in the susceptible age range. However, the vasculitis was also observed as late as 78 days.

C - Tissues

Details of the number and the class of tissues examined from the animals of susceptible age in groups "B" and "C" are summarised in table 10. A summary of the class and number of tissues examined at each age inoculation in groups "A", "B" and "C" is presented in tables 1 to 3 of Appendix II.

Within the susceptible age range the vasculitis was present in
six /

six tissues. In descending order of frequency these were: Brain, Mammary Gland (Fig. 23), Heart (Fig. 24), Pancreas (Fig. 25), Spinal Cord (Figs. 26 and 27) and adipose tissue. The latter was from the undeveloped mammary gland. The heart, pancreas and adipose tissue of the mammary gland were from a single lamb which was positive for vasculitis in the brain.

All of the affected blood vessels showed lesions of the same type as described in Chapter III. The only additional lesion noted was the segmental fragmentation of the intimal elastic lamina of the coronary artery in one lamb.

Special staining techniques added nothing to the information gained so far.

D - The effect of sex and litter size

There were 19 males and 9 females in the range of susceptible age (>82 days) from groups "B" and "C". The lesion was present in 3 out of 19 males and 2 out of 9 females. In the same age range 19 singletons and 9 twins were available for the study. The vasculitis was present only in the singletons (5 out of 19). Therefore it seems that the incidence might be higher in females than males, and singletons than twins. Details of the distributions of the sex and litter size of control and infected animals of groups "A", "B" and "C" in relation to the age at inoculation and the duration of post-inoculation are summarised in tables 4 to 8 Appendix II.

E - Assessment of the optimum CNS blocks for further studies

The optimum CNS blocks for the sampling in future studies, in descending order were: B2 (including corpus callosum, internal capsule etc.), B4a (Occipital pole), C7 (Cervical enlargement of spinal cord), B1 (Frontal pole) and B5 (Pons). Details are summarised in table 11.

Blood /

Blood vessels involved were the intergyral branches of anterior - middle - and posterior - cerebral arteries at B1, B2 and B3. The smallest branches (arterioles) of these vessels running into the brain substance were also involved. Parieto-occipital and posterior-temporal arteries were affected at B4a. The basilar artery was involved at B5 and B7. Sagittal sections of the cerebellum showed the lesion in the anterior and posterior cerebellar arteries. Sections of the spinal cord at C2, C7, T8 and L3 disclosed the involvement of the ventral-spinal and ventral-sulcal arteries, the branching arterioles of the latter artery and the branches of arterial vasa corona.

Sulci and fissures embracing the affected blood vessels were Sulcus ectolateralis, S. lateralis, S. suprasylvius, S. splenialis, S. posterior supra sylvian, S. posterior rhinalis and transverse fissure of occipital pole.

Within the brain substance affected arterioles were located in the radiatio corporis callosi, caudate nucleus, internal capsule, globus pallidus and hippocampus.

It was observed that the branches of an affected blood vessel may or may not be affected, and that symmetrically distributed branches of an artery may both show the lesion.

F - Non-specific findings

Macroscopic findings which might have indicated the presence of vasculitis were found in some animals in all three groups. These were:

Infarctions present in the kidney of three foetuses.

Nodular growths along the branches of omental arteries. These were whitish, firm, with depressed centre and 2-3 mm. in diameter. Peteshiae and ecchymoses were present in epididymis, testis, lung, epicardium, aorta, pancreas, ovaries and abomasum. In the latter the ecchymoses were sub-mucosal, more ^{gres} pronounced and had raised centres.

However /

However, in all these cases histological examination failed to reveal vasculitis associated with the lesion.

Other non-specific findings were: cystic transformations in the epididymis of a foetus. In lambs, oedematous and watery-looking brain, lobulated and undeveloped kidney, fibrinous pleurisy and adhesions of the lung to the chest wall, parasitic cysts in the liver, pancreas and duodenum, suppurative abscesses on the jugular vein, and finally, broken and healed ribs, were all observed.

III. DISCUSSION

In this experiment three groups of progeny were examined, i.e. groups "A", "B" and "C". Group "A" infected at 50th day of foetal life and sampled 5 to 10 days intervals thereafter. Groups "B" and "C" injected at ten day intervals through foetal life from 50th day. Group "B" were sampled at standard time (15 days) post-infection, whereas group "C" proceeded to term and lambs were sampled at three weeks of age. The experimental plan did not allow for larger groups of animals of identical status, but the programme of sequential sampling should reveal any trends.

From Chapter III it appeared that the predilection site for this specific vasculitis might be the CNS. However, the present experiment permitted the systematic examination of a wide range of tissues. Vasculitis was found in heart, pancreas, adipose tissue of mammary gland in addition to CNS which was confirmed to be the predilection site of the lesion.

The absence of the vasculitis in any control material, confirmed that its development is not a consequence of the injection of brain tissue per se, but results from the interaction of Border disease agent and host tissue. That this interaction is not a simple one is evident from the fact that no progeny of ewes infected at earlier than 82 days of foetal life developed vasulitis, whereas the lesion was present in those whose dams were injected at ≥ 90 days gestation. Ten days difference in the range of age susceptibility between the present experiment /

experiment and that suggested in Chapter III may be merely a reflection of the small numbers of individuals (i.e. one) in the 80 days sub-group. However, the occurrence of the vasculitis in the lambs infected at 130 days of gestation supports the hypothesis that the susceptible age range at inoculation extends from 82 days of gestation to at least the second day of neonatal life. Furthermore, it is suggested that the vasculitis has a monophasic developmental cycle. It would thus appear that the results of present experiments are in agreement with those of Chapter III, and that age at injection is an important factor in the development of this vasculitis.

Typical disseminated vasculitis was prominent in group "C" lambs whose mothers were injected at >90 days of gestation, but only one group "B" foetus of the same class (sampled 15 days post-inoculation) showed the lesion and then in a less advanced stage. It may be that in the latter group vasculitis was missed or that due to the small numbers the distribution appears skewed. A more probable and attractive hypothesis, however, is that survival time was insufficient for vasculitis to develop in this group. The presence of the lesion in one foetus of group "B" should not be assessed in the simple terms. As it is suggested by Silverstein et al. (1963a), immunogenesis is not merely an immunological process, but rather the by-products of a more general biological maturation or differentiation of multi-potential reticulo endothelial cells. Some individuals mature biologically earlier than the others. Hence, it might be explained that the presence of the lesion in one foetus of the susceptible age at 15 days after inoculation is possibly due to the premature development of this foetus. However /

However, in group "C" the minimum time interval between injection and sampling was 35 days. According to Silverstein et al. (1963a), the foetus is only slowly developing cells competent to respond to a variety of stimuli. Thus while the 90 to 100 day old foetal lamb requires 30 days to produce the first response to a given antigen it may take the 65 to 70 day old foetus some 60 days to accomplish the same result. Observations in Chapters III and IV (Table 9, Chapter IV and Table 5 Appendix 1), with respect to a minimum survival time required for the development of the lesion in relation to age at inoculation, show some trends to support Silverstein et al. (1963a) suggestion. However, due to the small number of the individuals in each sub-group and lack of serial sampling at a fixed interval at a standard inoculation time, only permits the conclusion that the shortest post-inoculation time to obtain the lesion is 21 days.

In the previous Chapter no effect of sex or litter size on the occurrence of vasculitis were observed. In the present experiments when only individuals in the susceptible groups are examined, i.e. ≥ 90 days at infection with survival time of > 15 days, four out of eight singletons had vasculitis compared with none out of four twins. This may be an indication of litter size influencing the development of vasculitis. Similarly, a sex bias was observed, three out of seven males against one out of five females showing vasculitis. These trends are in the opposite direction to those indicated in Chapter III.

The vasculitis-susceptible foetus differs from the adult on the one hand and the fully Border disease susceptible foetus on the other in /



in at least two important respects. It has susceptible tissue for the Border disease agent in the form of a myelinating CNS and further it has developed at least some immune responsiveness as in the sheep foetus, this begins to develop at about 60 days of gestation (Silverstein et al. 1963a). However, though the immune responsiveness starts much earlier in gestation than the lower limit of susceptible age - range suggested in this and the preceding Chapter, it is the rate of development of the reticulo-endothelial system which governs the consequent events. Hence the susceptibility toward different antigens commences at different developmental stages. Thus it is a reasonable speculation that vasculitis may be due to immunological responses. These might be due to:

- a. Humoral antibody produced against Border disease Ag(s) by an infected foetus or lamb. The outcome might be the formation of circulating Ag-Ab complexes in slight Ag excess and the localisation of these complexes in vascular structures. This is unlikely, however, as in such conditions one might expect to see acute glomerulonephritis, endocarditis, synovitis, splenic granulomas and cutaneous rash (Kniker & Cochrane, 1968), none of which have yet been seen in this study. Furthermore, in vascular reactions of the Arthus type the dominating cells of the infiltrate are neutrophils which were very scarce in the observed vasculitis.
- b. Cell mediated immunity evoked as a response of the primed lymphocytes against, possibly, the Ag(s) localised on or outside the affected blood vessel. This seems more likely since the majority /

majority of the infiltrating cells are of mononuclear series. If this be true the passage of the lymphocytes from the lumen across the blood vessel wall should be capable of demonstration.

In this context it may be significant that a tendency to bias with sex and litter size has been noted in this experiment. There is evidence that growth and possibly also development of twins, particularly after 80 days of gestation, is less advanced than in singletons (Winter and Feuffel, 1936). Differential rates of development between the sexes have also been shown for a number of parameters in a variety of species. Whether developmental parameters could be reflected in immunological terms is not known. However, it is possible that either the Ag may not be distributed evenly or it may be diluted to a level insufficient to produce vasculitis in twins.

IV. CONCLUSION

When the ewes were inoculated at appropriate gestational ages the earliest that the vasculitis ^{was clearly} developed in the offspring was twenty-one days post-inoculation.

CHAPTER V.

DETAILED MORPHOLOGICAL STUDY OF THE VASCULITIS:

LIGHT- AND ELECTRON-MICROSCOPY AND LIPID HISTOCHEMISTRY

| | Page |
|--|------|
| <u>INTRODUCTION</u> | 55 |
| A. <u>MATERIALS AND METHODS</u> | 56 |
| 1. Animals | 56 |
| 2. Procedure of anaesthesia, euthanasia and perfusion | 57 |
| 3. Post-mortem examination and sampling | 59 |
| 4. Fixation, preservation, processing and sectioning | 60 |
| 5. Staining | 62 |
| 6. Technique for the morphological study of the lesion by means of serial sections and the photographic montage | 64 |
| 7. Assessment of the class of affected vessels | 65 |
| B. <u>RESULTS</u> | 67 |
| 1. Animals | 67 |
| 2. Light microscopy | 69 |
| a. Plastic sections | 69 |
| b. Paraffin sections | 69 |
| 3. Histochemistry and immuno-chemistry | 71 |
| 4. Electron-microscopy | 72 |
| a. Type of cells | 72 |
| b. Cell migration | 73 |
| c. Necrotic debris, secondary lysosomes, residual and unidentified bodies | 74 |
| 5. Results of morphological study of the lesion through paraffin embedded serial sections | 75 |
| 6. Assessment of the class of affected blood vessels | 76 |
| C. <u>DISCUSSION</u> | 77 |
| D. <u>CONCLUSION</u> | 85 |

Using the information gained from previous Chapters, the experiments described in this Chapter were designed solely for the study of the vasculitis in Border disease. Hence, an attempt was made to reproduce the lesion in highest incidence, i.e. using ewes at the last third of their pregnancy in as large groups as possible and giving sufficient time for the lesion to develop. The major aim of the experiment was to study the lesion in greater detail using electron-microscopy, histo- and immuno- chemistry. Priority was given to the ultra-structural definition of the lesion and certain histochemical and immuno-chemical tests being held in reserve should the experiment yield sufficient material for their exploration.

A. MATERIALS AND METHODS

1. ANIMALS

Twenty-six pure Dorset-Horn females comprising 19 gimmers and 7 older ewes purchased from a farm with no history of Border disease were mated with a single raddled Dorset-Horn ram. The presence of the "Keel" mark and failure to return to the service determined the conception date. The pregnant ewes were divided in one small inoculum control group "A" (3 ewes) and two major experimental groups, viz. groups "B" and "C". Each of groups "B" and "C" contained 11 ewes, i.e. 2 controls and 9 infected. The remaining ewe conceiving much later in the breeding season was retained as a substitute should the need arise for an additional animal in either group "B" or group "C".

Nature, dilution, dose and route of inoculations of Border disease and control inoculum were as Chapter IV.

Group "A" was inoculated with infected inoculum on the 54th day of gestation and sampled 20 days later. Group "B" was inoculated on the 90th day and Group "C" on the 110th day of gestation. Both these groups were allowed to lamb and the lambs were sampled at between 4 to 6 weeks of age.

2. PROCEDURE OF ANAESTHESIA, EUTHANASIA AND PERFUSION

The techniques used for examination of the positive control ewes (group A) and fetuses, was as described in Chapter IV.

In lambs the autopsy technique was designed to obtain fixation of the brain and spinal cord to level of T2.

Terminology used is from Sisson (1953) and Miller et al (1964).

The perfusion apparatus was a 500 ml. blood transfusion bottle equipped with air-inlet and fluid outlet valves. The outlet was connected by a plastic tube to a drip chamber with filter. The drip chamber was connected to a perfusion needle via a plastic tube fitted with an adjustable clamp. The reservoir was hung 112 cms. above the artery to be perfused.

Live lambs were weighed and measured as described in Chapter IV. Lambs were premedicated with 0.1% heparin at a rate of 2 mg/5 lbs. of live body weight and anaesthetised with Pentobarbitone-Na given at a dose of 1 ml/5 lbs. of body weight. The depth of anaesthesia was assessed upon the respiration rate, pupillo-ocular and pedal reflexes. The subject was restrained in the supine position and a mid-line incision made along the anterior two-thirds of the sternum. The skin was reflected from underlying muscles by blunt dissection. The left anterior, posterior, superficial, pectoral and deep pectoral muscles and costochondral junctions of 2nd to 7th sternal ribs were divided. The left lobe of the pars thoracalis of thymus was reflected by blunt dissection and the common brachiocephalic trunk exposed. The left lung was pushed aside and an incision made on the left side of the pericardium exposing the heart and the thoracic aorta. The latter was freed from its attachments to the pericardium and the heart expressed from the pericardial sac, thus stretching the common brachiocephalic trunk and exposing it for insertion of the perfusion needle. The thoracic aorta was clamped at the point at which /

which the vena azygos crosses the arch using a pair of curved artery forceps, the jaws of which were guarded by plastic sleeves. A ligature was passed around the common brachiocephalic trunk and was held loosely by the assistant. Gently a 14 gauge needle, attached to the perfusion set, was inserted into the common brachiocephalic trunk about a quarter of an inch beyond its origin from the aortic arch at an angle of approximately 10° to long axis of the artery. The clamp of perfusion set was adjusted to obtain a full speed flow of the perfusate, a mixture of 1% glutaraldehyde plus 4% formaldehyde in 0.1 M phosphate buffer at pH 7.4. Once the perfusion had started the needle was tied in with the ligature and fixed with a further clamp. The anterior vena cava was then opened to avoid perfusion pressure rupturing small blood vessels. The right and left brachial arteries were dissected out and clamped at the point of exit from the thorax on the anterior border of the first pair of ribs. The jugular veins were located in the middle of the neck and opened, and the opening in the anterior vena cava sealed immediately with a clamp. The whole surgical procedure took three minutes. No artificial respiration was applied but care was taken not to puncture the right mediastinal pleura, which prevented the collapse of the right lung and allowed respiration to continue. The animal was breathing for one minute after the commencement of the perfusion. Five hundred mls. of perfusate was run in rapidly to clear blood from the vessels of the region. The rate of perfusion was then reduced to four drops per second. For each lamb 2,000 to 2,300 mls. of perfusate was used, the fixative running through the vessels for about 85 to 90 minutes. Twenty minutes after perfusion had ceased the clamps were removed and the post-mortem examination and sampling carried out.

Lambs for histo- and immuno- chemistry were killed by decapitation through atlanto-occipital space.

3. POST-MORTEM EXAMINATION AND SAMPLING

The post-mortem technique was the same for all of the animals. Perfused lambs were examined 30 to 40 minutes after completion of the perfusion. Freshly aborted fetuses, dead and unperfused lambs were examined as soon as possible after death. The cranium was opened through saw cuts leading from Foramen Magnum to the median canthus of the eye with a lateral curvature around the calvarium and the brain removed. The spinal cord was removed after cutting the vertebral arches bilaterally. Examination of the rest of the organs was as described in Chapter IV.

Blocks sampled from the CNS of the lambs varied according to the purpose which they were going to serve. However, some identical samples were obtained from the brain and spinal cord of all lambs as described in Chapter IV. These comprised B1, B2, B4a and C7.

One mm. thick blocks of CNS from perfused lambs served for EM studies. These were taken from sites adjacent to those shown to contain vascular lesions on H and E stained sections of the rapid paraffin blocks.

Corresponding blocks were obtained from the CNS of unperfused lambs for histo- and immuno- chemistry. In addition, samples were also obtained from liver and kidney for use as positive control material respectively for acid- and alkaline-phosphatase methods.

Brain and spinal cord were sampled further for routine paraffin sections. Since it had been shown that the vasculitis has a predilection for CNS and the lesion may show a symmetrical distribution, B1 and B4a were sampled from both cerebral hemispheres. Those organs in which vasculitis had been previously observed (Chapters III and IV) were also examined histologically. These included testes and epididymis (longitudinal block of both organs), lung (left apical lobe), mammary gland (a basal block of the organ where the arterial branches are more prevalent), sciatic /

sciatic nerve, heart (0.4 cm. below the coronary groove towards the apex), kidney (a frontal horizontal block), spleen (a transverse block 0.6 cm. from the hilus to include the splenic vessels), and pancreas (on the left extremity where the coeliac and anterior mesenteric arteries adhere to it).

4. FIXATION. PRESERVATION. PROCESSING AND SECTIONING

Fixation of tissues for rapid paraffin technique was in du Bosq's fixative at 37°C. Perfused tissues received 45 minutes treatment and fresh tissues two hours. Dehydration was in three changes of absolute alcohol each of thirty minutes' duration. The tissues were cleared in two changes of equal parts of absolute alcohol and toluene and one of pure toluene, each of thirty minutes' duration. Infiltration was done in a vacuum chamber through soft and fibro waxes with embedding in fibro wax.

Six micron sections were cut, as described for routine paraffin blocks in Chapter IV, stained H & E and examined the same day. Areas positive for vascular lesions were marked to locate suitable blocks for special study.

The perfused brains and spinal cords were preserved in a mixture of 1% Glutaraldehyde and 4% Formaldehyde in phosphate buffered solution at pH 7.4. For E.M. study, appropriate blocks were further serially sliced to 1 x 1 x 2 mm. and occasionally to 0.5 x 0.5 x 4 mm. pieces. These were washed twice in phosphate buffer solution pH 7.4 for 90 minutes, or occasionally overnight and post-fixed in 1% O_3O_4 for 90-120 minutes, washed in phosphate buffer with two changes each of twenty minutes. The blocks were then dehydrated through graded alcohols to two changes of absolute alcohol each of thirty minutes. Blocks were cleared in two changes of epoxypropane (Propylene oxide, BDH) each of fifteen minutes and then transferred to Araldite at room temperature. The following morning they were transferred to fresh Araldite and embedded at 60°C. for 72 hours. One micron section was cut on a Sorval "Porter Blum" Ultramicrotome for light microscopic examination. These /

Thin sections of 500-600 Å were cut on Ultratome 1 LKB 4800 and mounted on copper square grids of LKB 4 without any supportive membrane.

Fixation of material for routine paraffin sections depended upon the treatment the tissue had previously received. Those obtained from perfused animals were stored in perfusate. Tissues from the other sources, i.e. dead or perfused animals were fixed in Baker's (1944) calcium chloride formalin. Processing and sectioning were as Chapter IV.

Blocks for histochemistry were immersed in 4°C. cold Baker's fluid at pH 7.0 and fixed for 36 to 48 hours in a refrigerator at 2-4°C. Longitudinal blocks of spinal cord at C7 were fixed in Baker's fluid at room temperature for OTAN technique. Blocks for acid- and alkaline-phosphatase techniques were rinsed in cold distilled H₂O prior to sectioning. Serial frozen sections, at 12 μ were cut on the Frigistor Thermo-electric stage as in Chapter IV.

Samples for immuno-chemistry were put in polyethylene bags, labelled and quenched in a mixture of solid CO₂ and isopentane, and preserved at -80°C. Serial 6 μ frozen sections were cut in a cryostat at -25°C.

5. STAINING

The techniques used in this experiment were as in Chapters III and IV with the addition of:

a. Routine and special staining methods

- (1) Holmes (1947) silver technique for axons
 - (2) Giemsa for nuclear detail
 - (3) Gram-Weigert's modified (Conn et al. 1960) techniques for fibrin
 - (4) Gridley's (1953) technique for fungi
- Techniques 1-4 are from Drury et al., (1967)
- (5) Lendrum's (1949) Kiton-Red-Almond-Green (K.R.A.G.) for RBCs.
 - (6) Barlow's (1957) tri-basic stain for the granules of mast cells
 - (7) Trevan and Sharrock (1951) Methyl-Green Pyronin + Orange G for plasma cells

b. Lipid stains

- (1) Lison and Dangelie's Sudan Black B method for lipid (Drury et al. 1967)
- (2) Sudan IV for neutral lipids (Culling, 1963)
- (3) Lillie and Ashburn modified (1943) oil-Red-O technique for lipid
- (4) Smith-Dietrich modified (Cain, 1947), Nile-blue-sulphate for acidic lipids
- (5) P.A.S. reaction for glycogen
- (6) Pearse (1951) performic acid Schieff technique for unsaturated lipids
- (7) Adams (1961) perchloric acid naphthoquinone reaction for cholesterol and related substances (PAN)

Techniques 3-7 are from Bancroft (1967)

- (8) Adams (1959) OTAN for phospholipid, cholesterol and triglycerides
- (9) NaOH - OTAN (modified after Adams and Bayliss, 1963) for alkaline resistant phospholipids
- (10) O_3O_4 method for unsaturated lipids (Adams, 1959).

The reactions were checked at three and twenty hours.

(11) /

- (11) Cold acetone extraction + OTAN for cholesterol, cholesterol esters and triglycerids.

The block was extracted in previously cooled acetone for 48 hours in refrigerator. After extraction, the frozen sections were stained for OTAN technique.

Methods 8 - 11 are from Adams (1965)

c. Plastic sections for light and electron microscopy

One micron thick araldite embedded sections were mounted on slides in a drop of distilled water and dried at 60°C in the oven. Two techniques were used for the staining.

- (1) Toluidine-Blue and Thionine technique developed and used at the Anatomy Department of the Royal (Dick) School of Veterinary Studies as follows:

0.5g. toluidine blue

2.0g. thionine

100 mls. 1% borax sodium (aqueous)

Dissolve powdered dyes in borax solution. Filter before use.

Apply the dye to the sections and leave on the hot plate until the steam rises. Wash properly under running distilled water and examine.

- (2) Giemsa at a 1/10 dilution for 3-5 minutes in a Coplin jar at 60°C.

The stained sections were examined with a light microscope.

The corresponding blocks of the positive sections were

further used for thin sectioning. Sections of 500-600Å⁰

thick were stained by Reynold's (1963) lead citrate

technique. This stain was diluted in 0.01 N NaOH (1:19 v/v)

and grids were immersed for four minutes, washed in

running de-ionised distilled H₂O and dried in air.

Interrupted serial thin sections were cut at 1 μ intervals

to follow the pattern of the lesion and check its presence

at different depths. These were treated in the same way

as above. All grids were examined by Siemens 51 electron-

microscope at 50 K.W. Electron-micrographs were taken on

Ilford EM 5 and 6 plates at 1250 to 12500 magnifications

and printed at a further magnification of x3.

6. TECHNIQUE FOR THE MORPHOLOGICAL STUDY OF THE LESION BY MEANS OF SERIAL SECTIONS AND MONTAGE.

Sections stained for H & E were examined for the presence or absence of vascular lesions. The corresponding rapid or routine paraffin blocks of positive H & E sections were chosen. Slides were numbered serially and coated with egg albumin. Sections were cut at a fixed thickness and mounted on the corresponding numbered slide before cutting the second section. Sectioning and mounting were carried out all the way through the block until nothing was left of the tissue. Sections were stained for H & E and examined with Reichert Neo-Span microscope fitted with a drawing arm. Drawings were done either at x 40 or x 100 magnification for a set of serial sections. The scale corresponding to the chosen magnification was drawn at the top of the drawing paper. To keep the continuation and the deviations of the different images of a single blood vessel in the right direction, two points on the lumen of the injured blood vessels were chosen arbitrarily, i.e. 9 and 12 o'clock. The outlines of the lumen, media and the lesion were traced by three separate lines. All serial sections of a single block were examined and drawn in this way. A horizontal line was drawn for each row of drawings. On a tracing paper, a horizontal line was drawn and divided into 0.5 cm. divisions each representative of 6 μ thickness, and lines drawn at a right angle to each division and numbered. The horizontal line of the tracing paper was superimposed on that of the actual drawing. The point of the lumen situated at 9 o'clock was placed at a tangent to the vertical line. Then the outlines of the three coats were traced. Once the first image was drawn the tracing paper was moved so that the tangent point to the 9 o'clock position of the second serial section was superimposed, on the second vertical line keeping the horizontal lines /

lines also superimposed. The tracing paper was then moved to the third point and the process repeated. To avoid the difficulty in recognition of the continuity of representative lines of each coat, different colours were used. The parts of the outlines of the images lying underneath the second and subsequent images were traced by broken lines and the overlying ones by solid lines. To transfer the complete tracing to Bristol-Board for photography a horizontal line was drawn and the horizontal line of the tracing paper superimposed upon it. With a "Stylus Fine 79" pen the outlines of each layer were transferred separately to the Bristol-Board and inked in.

The scale on the top of the Fig5 applies to the diameter of the vessel. For clarity the scale in terms of the length of the lesion has been increased approximately one thousand fold.

7. ASSESSMENT OF THE CLASS OF AFFECTED VESSELS

Calibration of vessels according to lumen diameter, wall thickness and thickness of media was accomplished with the aid of a stage micrometer and an eye-piece graticule. One division of the eye-piece graticule was superimposed on that of a micrometer slide. For each objective magnification the number of sub-divisions of the eye-piece in one division ($1 = 100 \mu$) of the slide was calculated. Hence size of one sub-division of the graticule was known.

Since not all blood vessels were cut transversely and occasionally were broken, two different criteria were used:

a. Blood vessels cut transversely. Whether a blood vessel was cut at right angles to its long axis was decided on the following grounds.

- (1) circularity of the lumen.
- (2) uniformity of the endothelial cells for distribution and shape.
- (3) even thickness of the media all the way round.

The /

The thickness of the wall and diameter of the lumen were measured and the ratio worked out. Blood vessels with a luminal diameter of $\leq 100 \mu$ and a ratio of 1:1.7 to 1:2.7 were assumed to be arterioles.

b. For the assessment of vessels which were broken or were not cut at right angles the classification of blood vessels used in Chapter II (Figs. 6-9) was applied. Here the points considered were:

- (1) the ratio of the thickness of media to the wall thickness.
- (2) presence or absence of the internal elastic lamina.
- (3) density and the number of elastic lamellae in the media.
- (4) density and the number of the smooth muscle layers in the media.
- (5) presence or absence of the longitudinally located differentiated (smooth) muscle cells in the intima or adventitia.
- (6) presence or absence of the undifferentiated smooth muscle cells in the intima.

The assessment was carried out in the tissues of six lambs killed at approximately the same age. The distribution of the class of vessels in the sampled tissues of control animals was established and compared with the distribution of affected vessels in diseased lambs in order to determine whether the apparent predilection of vasculitis for CNS tissue was in any way related to particular classes of vessel.

B. RESULTS

1. ANIMALS

Thirty-four fetuses and lambs were obtained for examination.

Tissues of four aborted or dead-in-utero fetuses, two from each of groups "B" and "C" were not fit for histological examination.

Clinically, moderate degrees of specific neurological and cutaneous symptoms were present in five lambs. Four of these were from group "B" and one from group "C".

Satisfactory whole body perfusion of all of the fetuses of group "A" was obtained. Segmental perfusion in groups "B" and "C" was more variable. Very good fixation was obtained in 6/12 lambs. In 4/12 the results of fixation were reasonably good and in 2/12 unsatisfactory. The reason in one of the latter lambs was that the perfusion needle had punctured the artery in two places allowing the leakage of the perfusate into the thorax. In the other no reason for the poor fixation was found.

Histological examination of material obtained from the three ewes of group "A" revealed typical placentitis, and the four fetuses showed characteristic neuropathological changes of Border disease, thus confirming the infectivity of the inoculum.

Vasculitis was present in 10/12 group "C" animals infected at 110 days of gestation. The clinically affected lamb of this group was positive both for vasculitis and the hypomyelinogenesis of Border disease. However, vasculitis was not detected in any of group "B" animals i.e. infected at 90 days of foetal life.

Due to the variation in the duration of the pregnancy, time of abortion and neonatal death the post-inoculation time at sampling fluctuated between 56 to 92 days in group "B". In group "C" the time between inoculation and death varied from 29 to 79 days and the vasculitis was detected over the whole time scale.

The /

The data was examined to see if there was any effect of sex or litter size on the development of vasculitis, but none was found, as 4/4 females and 6/8 males showed the lesion and in terms of litter size 3/3 singletons and 7/9 twins had vasculitis.

A summary of the results in the animals of groups "B" and "C" are given in Tables 12 and 13 respectively.

2. LIGHT MICROSCOPY

a. Plastic sections

Examination of the thick plastic sections showed that the pathological changes were mainly localised to the adventitial layers of the arteries and arterioles (Figs. 36 and 37), though pyknotic or fragmented nuclei were also present in both adventitia and media which resembled those described in an earlier Chapter. However, the main constituents of the adventitial infiltrates were mononuclear cells of the lymphocyte - macrophage series. Mild oedema was also present in the arterial and arteriolar adventitia (Fig. 37). In some affected vessels a few lymphocytes were seen apparently attached to the endothelium with the main mass of the cell projecting toward the lumen (Fig. 37). Apart from this no pathological changes were recognised in the intima or endothelium.

b. Paraffin sections

A summary of the different classes of tissues sampled at various stages of post-inoculation from the lambs and fetuses of group "C" is given in Table 14.

Vasculitis was not found in any group "B" lambs though five were diagnosed as cases of Border disease on clinical and neuropathological criteria.

Ten out of twelve infected animals in group "C" had vasculitis and six of them were diagnosed also as positive cases of Border disease.

In descending order of frequency vasculitis was present (Table 14) in CNS (7/12) (Figs. 28, 29, 36 and 37), testes and epididymis (4/9) (Fig. 31), lung (3/11) (Fig. 32), heart (2/11) and kidney (2/11) (Fig. 33). In different blocks of CNS the distribution of the lesion in descending order was B4a (7/12), B2 (6/11), B1 (6/12), B5 (6/12), L3 (5/12), B4b (4/12), B6 (4/12), C2 (4/12), C7 (4/12), B7 (3/12) T8 (3/12).

Most affected blood vessels in the CNS were the intergyral ^apiarachnoid and vessels of the superficial brain substance.

With /

With two additions namely vacuolating change in the nuclei of the smooth muscle cells, and the presence of lipid droplets, between and in the cytoplasm of the infiltrated cells, the characteristics of the lesion were as described in Chapters III and IV. The lipid droplets will be discussed in detail under the next sub-heading. Other lesions were:

Focal symmetrical malacia and necrosis in B4a of one aborted group "A" foetus which was positive for both Border disease and vasculitis; similar lesions leading to cavitations were present at B2, B4a, B4b, B5, T8 and L3 of four lambs of group "B". These foci were round and circumscribed and surrounded by a rim of microglial cells. At the centre were some weakly basophilic filaments. These were translucent pink with H & E. Sections stained Gram's (greyish-pink), von Kossa's (pale pink), Gridley's PAS (yellow) and van Gieson (greyish-yellow) suggested that the filamentous material might be moderately mature or mature-amyloid, ^{however Congo Red method was negative.} This foetus and another lamb (9 of group "C") also showed thickening and swelling of the choroid plexus due to infiltration with fusiform cells. Both animals showed vasculitis.

Axonal degeneration with the formation of corpora amylacea was seen in the granular layer of the cerebellar foliae at B5 and ^{and the latter was seen} in the lumen of a capillary blood vessel at T8. These bodies were basophilic, occasionally lamellated and 20 to 100 μ in diameter. They stained positively with Holmes silver technique and von Kossa.

Lesions were observed also in spleen of five lambs (one of group "C" and four of group "B"). The group "C" lamb was positive for both vasculitis and Border disease. The splenic lesions were trabeculitis and capsulitis characterised by degeneration and infiltration by mononuclear cells. The walls of some arterioles in the lesion appeared weakly eosinophilic. However, sections stained with Fuchsin Miller, OTAN, G.S., Verhoeff and Congo red, all proved to be negative for fibrin, disorganisation of reticulin, elastic fibres or amyloid. No hypertrophy of blood vessel walls was detected.

Sections /

Sections of the liver from the lamb (No. 1 of group "B") with internal haemorrhage due to the rupture of the accessory lobe, did not reveal any vascular damage. The main findings were recent haemorrhage and coagulative necrosis of the hepatic cords. PER stained sections revealed huge amounts of haemosiderin in the haemorrhagic area.

Lungs and ileum of one lamb with necrotising pneumonia and ulcerative ileitis on gross pathology revealed fungal hyphae microscopically.

3. HISTOCHEMISTRY AND IMMUNOCHEMISTRY

No vasculitis was found in the blocks selected from unperfused tissue for enzyme histochemistry and immunochemistry.

In fixed tissues, application of OTAN technique confirmed the diagnosis of Border disease by demonstrating defective myelination and the accumulation of interfascicular lipid in fifteen cases. It further revealed the presence of lipid droplets in vacuoles in the adventitia and media of blood vessels showing vasculitis in five cases (Figs. 34 and 35). These lipid droplets were then explored histochemically by means of the methods listed in Table 15, from which it is concluded that the lipid droplets were composed of hydrophobic lipids probably cholesterol, cholesterol esters and triglycerides.

4. ELECTRON-MICROSCOPY

The method of selection of vasculitis containing tissues for E.M. was more successful than for histo- and immuno- chemistry.

The earliest ultrastructural changes observed were degeneration of the junctional zone between the media and adventitia and the infiltration of this zone by inflammatory cells. (Figs. 39-50). In some cases these changes were accompanied by distension of the extracellular space between the smooth muscle cells of the outer part of the media. The endothelium and basement membrane were intact and the smooth muscle cells resembled those in control individuals (Fig. 38).

Numerous inflammatory cells were trapped within a lattice work of collagen fibres (Fig. 46).

a. Types of cells

The infiltrating cells were classified according to Yamada and Sonoda (1970 a and b; 1972 a and b), and were of two types:

(1) Cells showing irregular contour with many small and large pseudopodic cytoplasmic projections. These had irregularly rounded or horse shoe-shaped single lobed nuclei (Figs. 40, 46, 48, 50) and a few had separated nuclear lobes (Figs. 39 and 47). The chromatin was distributed at the margins of the nucleus but its density was very variable (Figs. 40, 46, 47). A large nucleolus (Nu) was present (Figs. 45 and 46).

The cytoplasm contained numerous organelles. The number of mitochondria (M) was 9 - 23 per section per cell. The Golgi apparatus (G) was ill-developed, lamellar and located at the indentation of the nucleus (Figs. 46 and 48). Smooth endoplasmic reticulum (SER) was abundant and distributed as clumps of small round/or oval vesicles (Fig. 48), occasionally with a lamellar arrangement (Fig. 50). Rough endoplasmic reticulum (RER) was also present but in less abundance (Fig. 50). Free ribosomes and polysomes were present in the cytoplasm of almost every cell of this kind.

These /

These cells were identified as macrophages.

(2) Cells with more regular contours. Pseudopodic projections were absent (Figs. 39 and 40). The nucleus was clear and macular and had a narrow indentation and a poorly developed nucleolus. It occupied almost all of the cell.

The cytoplasm surrounding the nucleus was a thin rim. It contained SER which had vesicular arrangement in some of the cells and lamellar arrangements in others. RER was not very well developed but rudimentary formations were present in the cells with lamellar SER. In the latter type of cells 1 to 7 mitochondria per section were observed. These were located at the indentation of the nucleus and the side opposite it. The Golgi apparatus was ill-defined.

Cells with no RER and ill-defined mitochondria were identified as typical lymphocytes (TL), and the ones with RER and presence of mitochondria as atypical lymphocytes (AL) (Figs. 39 and 40).

No granulocytes (polymorphs, basophils or eosinophils) and plasma cells could be identified amongst the infiltrating cells.

b. Cell migration

Migration of the inflammatory cells from the lumen of the affected arteriole to the adventitia was suspected when the attachment of the lymphocytic cells to the endothelium was observed. Hence interrupted serial sections were studied. Migration is demonstrated through Figures 41 to 44.

Apparently the earliest phase of the migration was the attachment of the inflammatory cell to the cytoplasm of the endothelial cell. Once attached to the endothelium the inflammatory cell flattens (Fig. 41) and pushes out a cytoplasmic projection which invaginates the endothelial cell plasma membrane (Fig. 41). The process of invagination and devagination proceeds (Fig. 42) until the inflammatory cell becomes enclosed in the cytoplasm of the endothelial cell and then pushes through it. All of the intercellular tight /

tight junctions of the endothelial cells remain intact. Once the inflammatory cells pass to the media the process of the migration is apparently changed. Now, instead of running cytoplasmic processes into the cytoplasm of the smooth muscle cells, these pass through the junctions between the latter (Figs. 43 and 44 arrows). This process continues until the cells reach the adventitia.

Migrating cells were mainly recognised as lymphocytes, although the one cell which did not fit into the criteria of the lymphocytes is shown in Fig. 43. This cell has a large number of mitochondria and a well-developed Golgi apparatus.

c. Necrotic debris, secondary lysosomes, residual and unidentified bodies

Free or phagocytosed necrotic debris was present in the walls of affected blood vessels. Free debris (Fig. 45) was mesh-like or granular and of variable electron density presenting as particles measuring $6 \times 2 \mu$ and sometimes delimited with a pseudo-double membrane. These bodies were considered to represent pyknotic or degenerate nuclei of smooth muscle cells.

Phagocytosed debris in macrophages was of two types. One type (NC) had a well-defined delimiting membrane (Fig. 46), and was of oval shape with two shallow indentations on the equator and measured $2 \times 1.5 \mu$. This type consisted of two parts; a compact or dense region (a) which was finely granular; and less condensed, less granular part (b) which contained small vesicular bodies. The whole structure was considered to be a completely necrotic cell, about to lyse, possibly of infiltrated cell population. The other type of phagocytosed debris (NB) (Fig. 49) was oval and measured $4 \times 2.5 \mu$. It was dense and composed of fine electron dense granules and had no limiting membrane. This was also regarded as a necrotic cell.

Macrophages also contained secondary lysosomes in their cytoplasm which measured $0.5 - 4 \mu$ in diameter (Figs. 45, 47 - 49). Some of them were compact and filled with electron dense granules (Fig. 49). The limiting membrane was not easily distinguishable. Others which were less compact had /

had a well-defined limiting single membrane (Figs. 47 and 48) and contained either uniformly electron-dense granules (Fig. 47) or were filled with electron-dense (arrow) & electronlucent (double arrow) granules (Fig. 48). Dense granules measured 350 to 400 \AA and lucent granules 1300 \AA to 0.6 μ .

Residual bodies (RB) were also present in the cytoplasm of macrophages (Fig. 49). These had an ill-defined double membrane in which the inner membrane had extended to the centre producing pseudo-compartments, suggestive of mitochondria.

An unidentified body (UB) was located at the indentation of the nucleus of a macrophage (Fig. 50). This measured 1.3 x 2.3 μ and resembled a "morula". Ill-defined membranes criss-crossed and divided the interior into pseudo-lobules. There was a morphologic similarity between this body and orf virus. Though it is much larger than the latter (252 x 158 m μ) possibly it represents an extruded nucleolus.

5. Results of morphologic study of the lesion through paraffin embedded serial sections

The results are derived from eight paraffin embedded vasculitis containing blocks from different locations. They were from B1, B2, B4a, C8 and epididymis. The results in all locations were similar and an example from B4a is presented in Fig. 51a.

The diameter of the lumen and thickness of the media was unchanged. The lesion was mainly concentrated on the adventitia which showed segmental thickening. The distribution of the lesion in this coat was segmental, bilateral or unilateral and extended 60 to 282 μ in length. Occasionally lesions followed a regular sequence separated by 48 to 72 μ lengths of normal vessel. The nodular pattern of the lesion was observed properly through this study. Therefore it is suggested that the vasculitis in Border disease is a "periarteritis nodosa" without deposition of fibrin and disturbances of elastic membrane.

6. Assessment of the class of affected blood vessels.

A summary of the results is given in Table 16. Veins are not incorporated in the table since no class of these vessels was affected.

A total of 266 arteries and arterioles were calibrated in the tissues of control lambs, out of which fifty were transversely cut. In the affected lambs 245 of these vessels were calibrated of which 59 were cut transversely.

Different classes of arterioles and small distributing arteries comprised the majority of the blood vessels present.

Classes of affected blood vessels, in descending order of frequency, were large arterioles, small distributing arteries, medium and small arterioles. Large distributing arteries were not affected.

Cellular infiltrations increased the thickness of adventitia causing an overall increased thickness in the wall. As a result, the ratio of the wall to the lumen $\frac{W}{L}$ was increased to 1:0.7 in some arterioles. However, these could be identified as arterioles by the characteristics of their walls.

Perfusion had some effect on the measurements. This was apparently due to the contraction of the muscle layers and in all of the perfused tissues readings were higher for the luminal diameter and lower for wall thickness.

In order to get a measurement of the effect of perfusion, identical vessels of perfused and non-perfused normal control lambs were compared with respect to lumen diameter and wall thickness and a correction factor determined to off-set the effects of perfusion. This meant multiplying the wall thickness by two or three and dividing the value for lumen diameter by two. The variation in the figure for wall thickness was related to the quality of perfusion; in well perfused animals the higher figure was used for correction.

This correction factor used throughout assuming that vessels with vasculitis would be affected by perfusion to about the same extent as normal vessels.

C. DISCUSSION

Two groups of animals derived from the ewes inoculated at 90 and 110 days of gestation respectively, were used for the reproduction of vasculitis with the Border disease agent. Vasculitis was obtained in ten out of twelve of the animals of only one group, i.e. those whose mothers were infected at 110 days of gestation. This discussion centres upon these animals.

Electron-microscopy revealed that the lesion was formed by the infiltration of mononuclear cells mainly typical and atypical lymphocytes and macrophages into a degenerate zone at the interface of the media and adventitia. The macrophages phagocytosed the products of degeneration with the formation of secondary lysosomes and residual bodies.

The presence of the secondary lysosomes in the cytoplasm of the macrophages is suggestive of enzymatic activity (Novikoff and Holtzman, 1970). Lysosomes have a dual function. They digest the phagocytosed intracellular debris, and disruption of their delimiting membrane releases hydrolytic enzymes which are noxious for the tissue and cause further damage (Miescher *et al*, 1964). Hence it seems plausible to suggest that the necrosis in the wall of affected vessels is due to invasions by primed lymphocytes of the sites where antigen is localised, and subsequent release of the lysosomal hydrolytic enzymes. Migrated lymphocytes might react with antigen in the blood vessel wall and cause necrosis. Lysosomes then digest the necrotic debris, disrupt and release hydrolytic enzymes, which causes further tissue damage and attract macrophages. This inter-relation would explain the longstanding nature of the lesion and the absence of a healing stage. However, one would expect a progressive lesion to be the outcome of this pattern, but in the light of the observations referred to in Chapter III, progressive and healing stages were apparently absent in the vascular lesions of the epididymis of the male lamb examined six months post-inoculation.

Migration /

Migration of the inflammatory cells across the post-capillary venules has been demonstrated in the lymph node, spleen, brain and other tissues in the course of bacterial and viral infections (Marchesi and Gowans, 1964; Åström et al. 1968; Barlinger and Griffith, 1970; and Doherty, Reid and Smith, 1971). According to these scientists lymphocytes and plasma cells traverse endothelial cytoplasm, whereas monocytes and polymorphs pass through the inter-endothelial cell junctions. Whatever the route of migration no recognisable injury to endothelium results. As has been suggested by Marchesi and Gowan (1964) and Åström et al. (1968) the first stage of the traverse is the attachment of the mobile lymphocytes to the endothelial cell. The lymphocyte then flattens, projects into the endothelial cytoplasm, and becomes enclosed. It then pulls itself out of the cell into the sub-endothelial tissue. The findings in the present work accord with this view. However, there were minor differences.

1. The blood vessels which have been traversed are arterioles. This site has not been reported hitherto.

2. Once the lymphocyte has passed through the endothelial cell into the sub-endothelial tissue its mode of penetration changes. Instead of traversing the cytoplasm of the smooth muscle cells it pushes between the intercellular junctions. This change of the method of migration has not been reported previously.

The change of the method might be due either to the change of milieu or to transformation of lymphocytes to immunoblasts (Medawar, 1965), plasma cells (Turk, 1967) and macrophages (Nelson, 1969). However, according to Yamada and Sonoda's (1970 a and b, 1972 a and b) classification, almost all of the observed cells were lymphocytes and macrophages. According to their work each lymphocyte contains 3 to 7 mitochondria per cell and a poorly developed Golgi apparatus. However, one of the lymphocytes (Fig. 43) showed a well-developed Golgi apparatus and a larger number, i.e. 12, of mitochondria. This was a deviation from the classification used but accords with the classification /

classification of lymphocytes applied by Marchesi and Gowan (1964).

Yamada and Sonoda's (1970 a and b, 1972 a and b) work was on the peripheral leucocytes and whilst in the circulation these cells do not require much energy to move around. Once in passage across a thick arterial wall the energy requirement would be expected to be much greater, and more than that required to traverse the wall of a post-capillary venule. To produce energy, lymphocytes have to use carbohydrates, ATP and oxidative enzymes present in the mitochondria. This may be the explanation for the increased number of mitochondria observed.

Doherty, Reid and Smith (1971) demonstrated that the predominant cell in the population of the perivascular cuff in Louping-ill (L.I.) was the plasma cell, rich in RER, and capable of producing antibody (Ab) against L.I. virus in the brain. Cunningham, Smith and Mercer (1966) demonstrated that polyribosomes, in the absence of RER, can produce antibodies (Abs). Plasma cells were absent amongst the population of inflammatory cells in the peri-arteriolar space and the infiltrated cells were devoid of fully developed RER. Also, the polyribosomes were dispersed in the hyaloplasm. Hence, all the present evidence opposes the hypothesis that the cells in the vascular adventitia are concerned with free Ab production.

Cochrane and Aikin (1966) demonstrated the role of neutrophils (PMN) in immunological reactions. Through in vivo studies they showed that in Arthus' phenomenon, glomerulonephritis and nephrotoxic nephritis, the PMNs are responsible for the destruction of the basement membrane and the internal elastic lamina. Amongst the PMN enzymes (acid-and alkaline-phosphatase, lysozyme, lipase) only cathepsin was incriminated strongly as a cause of the destruction. However, other factors such as the interaction of complement (C) reacting /

reacting with immune complexes, or permeability factors released as the result of antigen-antibody (Ag-Ab) complexes should not be ignored. Attempts to demonstrate complement fixing (CF) and circulating Abs in association with Border disease have failed so far. In the present study PMNs were not evident and would thus explain the absence of disruption and fragmentation of the basement and elastic membrane.

The presence of the macrophages and lymphocytes in the adventitia and peri-adventitial space (Vircho - Robin space) raises questions about origins of these cells and the factors governing their transportation to the media-adventitial interface. They may be endogenous from Hortege cells and pericytes as suggested by classical studies of Rio Hortege (1932) or exogenous from haematogenous sources. Studies on the origin of the macrophages in damaged mice and rabbit brains by ^3H -thymidine and carbon tracers have ruled out the possibility of Hortege cells (Koni~~g~~smark and Sidman, 1963; Huntington and Terry, 1966; Stenwig, 1972; Adrian and Williams, 1973 and Kitamura, 1973). These workers have shown that macrophages infiltrating to the injured brain are derived from the blood mononuclear cells migrating across the blood vessels. As mentioned earlier, migration can occur either via venules or arterioles. In the former condition it is plausible to assume that after traversing the venules the macrophages, by means of their pseudopodic movement, can travel along the Vircho-Robin spaces and reach the injured area. However, evidence provided in this work is not in favour of this and though tracer elements were not used, the E.M. study is in accordance with the haematogenous route across the arterioles walls. Therefore, it is suggested that the lymphocytes and macrophages, present in the adventitia of the affected blood vessels are derived from the blood mononuclear cells.

In recent years it has been shown that activated lymphocytes produce molecular mediators called lymphokines which have different potentialities and /

and exert a variety of effects on macrophages (WHO, 1973). One of them is macrophage chemotaxis due to chemotactic factors (MCF). Considering the factors governing the migration of macrophages to the site of the lesion MCF seems likely to be implicated. Possibly activated lymphocytes traverse the blood vessel wall to the site where the Ag(s) is located. There they produce MCF which attracts the monocytes from the blood stream to the adventitia of affected blood vessel.

These observations provide evidence that the vasculitis in Border disease is an immunological response which may be associated with cell mediated immunity.

Takebayashi (1970) inducing four different types of hypertension, studied arteriolar lesions with E.M. He suggested that the major components of the arteriolitis were "focal cytoplasmic necrosis" of the smooth muscle cell, leading to fibrinoid necrosis, and "moth eaten structures". On the other hand, Scott et al. (1966 and 1967) have shown that the changes in the atherosclerosis of Rhesus monkey, as well as of man, are confined to the fibroblastic series of cells, and lead to the maturation and proliferation of new smooth muscle cells. In this study smooth muscle cells were usually intact. The changes were minor, mainly nuclear, and did not resemble the changes observed in either hypertensive arteriolitis or atherosclerosis. Thus, the possibility of a hypertensive vasculitis induced by a hypothetical release of histamine and associated chemotactic factors is improbable.

However, further studies directed towards the immunological and histochemical aspects of the lesion may throw more light on the nature of the immunological response.

Storey and Barlow (1972) in their study on the nature of the lipids in Border disease affected lambs, demonstrated the presence of the lipid droplets in the perivascular spaces. These workers, as well as Barlow and Dickinson (1965) demonstrated that the lipid is composed of hydrophobic lipids, probably triglycerids /

triglycerids, cholesterol esters and possibly free cholesterol. The results obtained in this work is in accordance with their findings in terms of the nature of the lipids. However, considering the class of blood vessels and the site of lipids location there were differences between their findings and present work. The lipid droplets according to Storey and Barlow were located in the lumen and perivascular spaces of the venules, whereas in this work demonstrated lipid was located in the cytoplasm and in between of the macrophages infiltrated to the media and adventitia of arterioles affected with vasculitis.

Paterson in his review article (1959) stated that complement fixing (CF) antibrain Abs appear in animals following repeated injections of heterogenous nervous tissue extracts or after a single injection of homologous or heterologous nervous tissue combined with adjuvant. Harwin et al. (1961) demonstrated that the sera from sensitised animals fix complement (C) with ethanolic extracts of their own brain suggesting that CF antibodies react as autoantibodies. In earlier studies, Thomas et al. (1950) had demonstrated that the Abs are directed against antigenic constituents of nervous tissue. These Abs resembled lipids, being insoluble in water but soluble in different organic solvents including ethanol. These findings raise the question that the vascular lesions of Border disease and the presence of the lipid may be due to allergic encephalomyelitis, the lipid droplets being possibly CF-antibrain-Abs. This cannot be the case since:

1. There is species difference and production of allergic encephalomyelitis in sheep is extremely difficult (unpublished data by workers at Moredun).
2. The inoculum used for the reproduction of the vasculitis with the Border disease agent was not combined with adjuvant.
3. A single dose of the inoculum was used.
4. Lipid droplets were absent in the control lambs born to ewes inoculated with normal brain suspension.

Therefore /

Therefore it is suggested that the lipid droplets may be due to minor medial degenerative changes, disruption of lysosomes and release of their by-products.

Calibration of blood vessels was undertaken to determine whether vasculitis predominantly affects certain types of vessel.

Calibration showed that there was no difference between affected animals, with or without vasculitis, and controls with respect to blood vessels in the blocks selected for study. Therefore, the class of blood vessel cannot be the reason of this predilection, as then the lesion should have wider distribution. It was found that four classes of blood vessels are affected i.e. small distributing arteries and large, medium and small arterioles, the highest occurrence being in the first two types. Hence, it would seem that the presence of Border disease agent in the brain and the availability of particular types of susceptible blood vessel may be the factors influencing cellular infiltration. In other words, interaction of three factors, viz. organotropism of the agent, class of blood vessel and immune responses may give rise to the lesion.

Involvement of the veins in vasculitis is reported by Harvey et al. (1954), Bergstrand (1946), Anderson & Vogel (1961). In the present work venous involvement was never observed and it is suggested that the vascular lesions associated with Border disease are exclusively arterial and arteriolar.

There is enormous literature on periarteritis nodosa in man and animals. Hitherto the condition has not been studied in depth in sheep and predilection sites for this lesion have not been established. However, Zeek's valuable review et al of 1952, and the work of Harris et al. (1939), Jones et al. (1957), Drake (1964), Miller et al. (1965) and Bruetsch (1971) indicates that in most species and conditions the predilection site of the periarteritis is not CNS. According to the literature the main organs involved are kidney 85%, heart 70%, liver 65%, gastro-intestinal tract 58%, spleen 31%, muscle 30%, cutis 25%, peripheral nerves 20% and CNS 8 to 25%. In Border disease infection, however, the predilection /

predilection site of periarteritis is CNS which may be a reflection of the neurotropism of the causal agent and/or the localisation of immune complexes in blood vessels of target organs. However, the other tissues which showed significant vasculitis were testes and epididymis (4/9). Vasculitis does not appear to have been reported previously in these tissues.

Ball and Dawson (1949) in their studies on the splenic changes in periarteritis nodosa, divided their material into four categories, namely:

1. in which non-specific lesions were present
2. in which typical vascular lesions of periarteritis nodosa predominated
3. in which capsulitis, trabeculitis and Malpighian body lesions co-existed with the typical vascular changes of periarteritis nodosa
4. in which trabeculitis and Malpighian body lesions were present, while the typical vascular manifestations of periarteritis nodosa were either negligible or absent.

They concluded that trabeculitis and Malpighian body lesions were usually associated with lesions of periarteritis nodosa, but from a study of serial sections, they found that the former could occur without the association with vasculitis.

Trabeculitis and capsulitis in the spleens of five lambs from the present study relates to the third and fourth categories of Ball and Dawson (1949). In one lamb of group "C" this lesion corresponds with the third category of these workers whilst the four lambs of group "B" agree with their fourth category. It is possible that the course of the disease in the four group "B" lambs giving rise to the inflammatory changes in the capsule and trabeculi in the absence of periarteritis has been very acute. If this is so, then it would seem that the generative mechanisms of Border disease associated periarteritis are functioning at ninety days of gestation in D.H. lambs, even though overt vascular lesions were not observed in this group of lambs.

The choroiditis observed in two lambs of group "C" may be a further indication of an early inflammatory reaction against localised Border disease agent or circulating immune complexes.

Compared /

Compared with the results discussed in Chapters III and IV, the absence of vasculitis in the progeny of the ewes inoculated at 90th day of gestation is of interest. Considering all factors in the experiments, it seems that the difference may be due to breed differences.

In group "C" animals the lesion was slightly more frequent in females (4/4) than in males (6/8). This finding contrasts with the pooled results of Chapters III and IV, i.e. males 10/17 and females 7/21. The difference is of doubtful significance and may again be related to breed.

D. CONCLUSION

Using the information gained from Chapters III and IV, several cases of periarteritis have been produced experimentally. The light and E.M. distribution and appearance of the lesion is described. The lesion consists of minor degenerative changes, i.e. nuclear fragmentation and mild oedema, and infiltration of macrophages and typical and atypical lymphocytes originating from blood mononuclear leucocytes. Hydrophobic lipid droplets were found between and within the cytoplasm of infiltrating macrophages. Reconstruction of the lesion through the drawings of serial sections have revealed its nodular, segmental nature. Large arterioles and small distributing arteries were found to be the main classes of affected vessels. It is concluded that the lesion probably results from immunological mechanisms.

IMMUNOLOGICAL AND HISTOCHEMICAL STUDIES OF THE VASCULITISCONTENTS

| | Page |
|--|------|
| INTRODUCTION | 87 |
| A. MATERIALS AND METHODS | 87 |
| 1. Animals, inocula, routes, euthanaesia and sampling | 87 |
| a. Ewes | 87 |
| b. Lambs | 87 |
| (1) - Skin test | 87 |
| (2) - In vivo macrophage adherence test | 88 |
| (a) - Lambs | 88 |
| (b) - Guinea-pigs | 90 |
| (c) - Cell count | 90 |
| c. Rabbits | 91 |
| 2. Immunology | 91 |
| a. Sera | 91 |
| (1) Preparation of rabbit anti-sheep fibrin serum | 91 |
| (2) MF 11 | 92 |
| (3) Sheep hyperimmune serum against Border disease | 92 |
| b. Tissues, sectioning and staining | 93 |
| 3. Enzyme histochemistry | 94 |
| a. Tissues and fixation | 94 |
| b. Sectioning | 95 |
| c. Staining | 96 |
| B. RESULTS | 96 |
| 1 - Animals | 96 |
| 2 - Immunology | 97 |
| 3 - Histochemistry | 100 |
| C. DISCUSSION | 102 |
| D. CONCLUSION | 106 |

IMMUNOLOGICAL AND HISTOCHEMICAL STUDIES OF THE VASCULITIS

INTRODUCTION

The results of the previous three chapters indicated that the Border disease agent induced vasculitis is an immunological response. The present experiments were designed primarily for the study of immunological aspects of the lesion, but were such that the nature of the infiltrative cells and the minor degenerative changes could also be examined histochemically.

A - MATERIALS AND METHODS

1 - Animals, inocula, routes, euthanaesia and sampling

a. Ewes - Production of vasculitis in lambs - Thirty-eight Cheviot ewes purchased from an approved Border-disease-free farm were mated with a Dorset-Horn tup. Conception dates were determined as described in chapter V. Pregnant ewes were divided into two groups "A" and "B". Group "A" contained 15 ewes, of which 3 served as controls. Group "B" comprised 23 ewes, i.e. 7 controls and 16 infected. Group "A" was inoculated at 90 days gestation and group "B" at 110 days of pregnancy. The route of inoculation and the inocula were as in chapter V. Both groups were allowed to lamb and lambs were sampled between 3 - 6 weeks of age.

b. Lambs

(1) Skin test - Nineteen lambs infected and thus sensitised in utero, through s.c./i.p. inoculation of mother were used for this purpose. Five were born to ewes of group "A" infected with Border disease inoculum and the rest were from group "B", i.e. 7 controls and 7 infected.

A $9 \times 9 \text{ cm}^2$ area was shaved on both sides of all lambs and divided into four $4.5 \times 4.5 \text{ cm}$ regions using a felt-tipped pen. The thickness of the skin in each of the smaller squares was measured with a skin caliper and recorded.

Two inocula of Border disease agent were prepared in a dilution of 1/200 in sterile saline. One of these was treated overnight with di-ethyl-ether at 2-4°C. The other was heated at 60°C for 90 minutes. Similar treatments were applied to inocula made from normal brain.

Antigenicity of the Border disease inocula treated with di-ethyl-ether was assessed on the basis of the results obtained from the fluorescent staining of the Border disease infected monolayers with an anti Border disease serum. Monolayers were fixed for 10 minutes in acetone and treated with di-ethyl-ether for a further 15 minutes. These treatments did not diminish the specific fluorescence. The antigenicity of the heat-treated inoculum was assumed on the basis of the findings of Gardiner et al (1972). A 0.1 ml dose of each inoculum was injected intradermally (i.d.) at its pre-determined site on each side of the animal.

To test the immediate effect of the inocula on the vascular permeability, three lambs, two sensitised at 110 days gestation, i.e. a control and an infected, and one sensitised at 90 days gestation received 6 mls 2% Evans blue dye in sterile normal saline i.v. The responses were checked immediately after injection and 3, 6, 24, 48 and 72 hours thereafter.

Skin specimens from each site were sampled for histological examination at autopsy, 12-26 days post i.d. inoculation.

2 - In vivo macrophages adherence test

(a) Lambs - Fifteen group "B" lambs, 7 controls and 8 infected, sensitised in utero via s.c./i.p. inoculation of mother were used for this purpose. All except one had also been used for the skin test.

Four /

Four days before slaughter, 20 mls sterile liquid paraffin was injected i.p. to produce a peritoneal exudate. One to two hours before killing 3 mls of 1/200 Border disease inoculum was injected i.p. Control lambs received the same treatment using normal brain suspension.

Lambs were weighed and anaesthetised with Pentobarbitone sodium as described in chapter V. The animal was restrained in the supine position. A site about 2 cms to the left of Linea alba was shaved. An incision was made and the skin detached from its underlying tissues by blunt dissection. The incision was enlarged to a length of 6-7 cms. The edges of the cut were held higher than the underlying tissues by means of 2 pairs of surgical tissue forceps to prevent leakage of blood into the area. A second incision was made through the Linea alba and peritoneum. An assistant fixed two pairs of surgical tissue forceps to the edges of the cut, this time holding all the tissues at once i.e. parietal membrane of the peritoneal sac, muscle layers and skin. The first two pairs of forceps were replaced and put in the same position as the latter ones. All of the forceps were held high to produce a deep cavity.

Multipor^e needle caps made from disposable needle caps were fixed to twenty mls syringes loaded with 19 gauge needles. The syringes were filled with 3% normal sheep serum in Hank's solution containing 5 i.u./ml heparin. One hundred mls of this was sprayed into the peritoneal cavity and the abdomen gently kneaded for 5 minutes. The washed exudate was harvested with the same apparatus and collected in sterile plastic universal bottles, for total, differential and absolute cell counts.

The animal was killed by decapitation through the atlanto-occipital space. The brain and spinal cord were immediately removed by the technique described in chapter V and specimens taken for histo-chemistry and immuno-chemistry.

(b) Guinea pigs - Ten 400-450 grs. female albino guinea pigs were divided into 2 groups of 5. One group was sensitised i.p. with 2 mls 1/200 Border disease inoculum 6 weeks before killing and the other group treated in the same way with normal brain suspension. Four days prior to killing both groups received i.p. 10 mls of 0.02 mg/ml sterile Oyster Glycogen (BDH) in physiological saline. Each group was challenged i.p. with 1 ml of its appropriate inoculum 1-2 hours before killing, i.e. normal brain inoculum and Border disease infected inoculum.

The animals were anaesthetised with ether, restrained in the supine position. The abdominal skin was dissected and 20 mls of 3% normal guinea pig serum in Hank's containing 5 i.u./ml. heparin was injected i.p. The abdomen was kneaded for 5 minutes. The abdominal wall was opened on Linea alba and the edges of the cut held higher than internal organs, as described for lambs, and the exudate harvested. No other tissues were sampled from guinea pigs.

(c) Cell count - Harvested exudates were used for total and differential-cell-counts. Absolute differential values were then calculated. The techniques of counting were similar both for lambs and guinea pigs, the only difference being the volume of the exudate available for centrifugation and preparation of the smears.

The exudate was diluted x 10 in the standard pipette using white cell diluent. Total cell counts were done using both chambers of an improved Neubauer haemocytometer. The cells were recounted by an independent assistant. The total of the readings of the two chambers by both individuals were divided by 4 and the values multiplied by dilution index i.e. 25.

For differential counts, some of the exudate, 20 mls for lambs and 10 mls for guinea pigs, was centrifuged at 100 g. for 5 minutes and the precipitated cells /

cells re-suspended in 0.2 mls of 50% normal sheep or guinea pig serum in Hank's solution. Smears were made from the cell suspension and dried in air at room temperature. Some of these were fixed in absolute methyl alcohol for one minute. Smears were stained Leishman, examined under oil immersion and the cells counted using a battlement system of traversing the edge of the film.

c - Rabbits - Two 3 Kg female albino rabbits were used for the production of rabbit-anti-sheep-fibrin-serum (RASFS).

2 - Immunology - a - Sera:

(1) Preparation of RASFS - Sheep fibrin was prepared according to Vasquez and Dixon (1958) with some modification.

Citrated blood was obtained from a normal Cheviot sheep and 1 M CaCl_2 at a rate of 0.025 mls/ml of plasma added to clot the fibrin. The washed clot was centrifuged twice, freeze dried and reduced to fine powder.

Thirty mgs. powdered fibrin per animal was suspended in Freund's incomplete adjuvant and injected into rabbits at weekly intervals by i.m. and s.c. routes alternately for 10 weeks. The rabbits were bled 4 weeks after first injection and thereafter at weekly intervals with bleeding out after the last injection. Blood was taken 3 days after each injection for detection and assessment of the concentration of antibodies.

Antibodies were detected by immuno-electrophoresis and gel diffusion using normal sheep serum (NSS) and normal sheep plasma (NSP) as antigens. After the fourth injection antibodies against fibrin were demonstrable as were unwanted antibodies against other serum components.

Small amounts of antisera were absorbed with doubling dilutions of NSS and the mixtures examined for the presence or absence of unwanted antibodies. Antisera were then absorbed in quantity using the optimum amount /

amount of NSS determined in the preliminary test. After incubation at 37°C for 30 minutes and at 4°C overnight precipitated antibodies were removed by centrifugation at 14000 r.p.m. for 30 minutes. The resulting absorbed antiserum gave a fibrin precipitin line with plasma but no lines with serum.

Absorbed serum was fractionated on a Diethyl-amino-Ethyl (DEAE) cellulose column in 0.01 M PO_4 pH 7.6 buffer. The resulting fractions were tested for the presence of rabbit IgG by immuno-electrophoresis and double gel diffusion using a sheep anti-rabbit IgG serum. IgG containing fractions were pooled and concentrated to give a protein concentration of 12-14 mg/ml as determined by spectrophotometer at 280 m μ .

For conjugation with FITC the protein concentration was adjusted to 10 mg/ml with pH 9.5 carbonate/bicarbonate buffer. Twenty five micrograms FITC per mg protein was added and the mixture stirred at room temperature for one hour. Unconjugated dye was removed by passage through a Sephadex G-25 column equilibrated with IXPBS. Conjugated serum was absorbed on rabbit liver powder for further purification.

Control tissue, i.e. the fibrinous exudate of the pleura of a lamb inoculated with Past. haemolitica was stained with different dilutions of the conjugate to determine the optimal dilution. This was then used to detect any fibrin deposition on the affected blood vessels' wall.

(2) - MF11 = Commercial rabbit anti-sheep immunoglobulin conjugated with FITC (Wellcome Reagents, Ltd.)

(3) - Sheep Hyperimmune serum against Border disease (HSABD). This was kindly prepared and provided by Mr. A.C. Gardiner of Moredun Institute. The technique used is briefly mentioned here.

Ewes which had given birth to severe hairy shaker lambs as a result of experimental infection with Border disease, were "hyperimmunised" one month after /

after parturition as follows. A dose of 3 mls 1/10 dilution of Border disease brain/spleen in Freund's complete adjuvant was injected at three i.d. and one i.m. sites. This was repeated 5 days later. After a further 2 months they were given another "booster" dose and bled a few days after the last injection. The sera stored at -20°C .

The sera were fractionated and conjugated by the above methods, were tested against foetal lamb kidney cells in tissue culture infected with Moredun Institute Border disease isolate. The agent was detected by immuno-fluorescence using this serum at serial dilutions to 1/128. For the detection of antigen in blood vessels affected with vasculitis, this antiserum was used at a dilution of 1/64.

b. - Tissues, sectioning and staining

Brain and spinal cord of all lambs killed fresh were sampled. The CNS was sampled at the levels of B1, half of B₂, B4a (from right cerebral hemisphere) as shown in Fig. 22 and C7, T8 and L3. Samples were put in polyethylene bags, quenched and stored for later use as described in chapter V. As some of the blocks, such as B₂ and B4a, were larger than the surface of the tissue holder used in cryostat microtome, they were divided further into 2 or 3 smaller parts. Six or seven sets of 21 slides, according to the number of blocks to be sectioned, were numbered serially and labelled with a diamond. Twenty-one 6 μ serial sections were cut on the rocker microtome of a cryostat as described in chapter V, sections 1, 6, 11, 16 and 21 fixed in absolute methyl alcohol for 5 minutes and stained H & E. The remaining sections were dried under the air current of a fan for 10 minutes, fixed in acetone for a further 10 minutes and left to dry for 10 minutes. These were then stored at -20°C before use. Four sections of each tissue sampled from control animals were cut and treated as above. No control sections were stained for H & E.

Haematoxylin /

Haematoxylin and eosin stained sections of infected animals were examined for vasculitis. Acetone fixed unstained sections preceding and succeeding any positive H & E section along with sections of tissues from control animals were used for immuno-fluorescent staining.

Direct and indirect staining methods were applied for the demonstration of antigens localised on the affected blood vessels wall. For direct staining conjugated HSABD was used. For indirect technique two sera were used, unconjugated HSABD being followed by MF11.

For the demonstration of fibrin deposition positive sections, selected as above, were stained along with positive control sections by the direct technique.

Stained sections were examined with a Leitz U.V. microscope equipped with an HB200 lamp using green K510 edge filter and CB16.5 holder filter. Photographs were taken with Leitz Orthopan automatic camera using Ilford PAN-F for black and white prints.

3 - Enzyme histochemistry

a. Tissues and fixation - Specimens were sampled from the CNS and liver immediately after exsanguination of the lamb through decapitation. Two sets of blocks were sampled as the techniques to be used required different fixatives. All CNS blocks were sampled from the left cerebral hemisphere at the levels of B1, B₂, B4a, C7, T8 and L3, (as described for immunochemistry) and a 10 x 10 x 3 mm slice of liver was also taken. The first set of the CNS blocks was taken from position contralateral to those sampled for immunochemistry. These were fixed in cold neutral Baker's fluid for 36-48 hours and used for demonstration of acid-phosphatase, alkaline-phosphatase and β -glucoronidase activities. A second set of identical /

identical unwashed blocks was sampled from the adjacent interface of the first set and fixed at 4°C in neutral formal saline for 96 hours for the demonstration of aryl-sulphatase activity. Liver was used as positive control for all of the techniques.

b. - Sectioning

(1) For acid-phosphatase, alkaline-phosphatase and β -glucuronidase techniques - Four sets of 26 slides were prepared and marked 1, 6, 11, 16, 21 and 26. Twenty-six 12 μ serial sections were cut on a freezing microtome from each block. Sections 1, 6, 11, 16, 21 and 26 were put in Petri dishes containing distilled H₂O marked for each number. Sections following the interrupted ones e.g. 2-4 or 7-10 were put in jars marked for intermediate sections, containing neutral Baker's fixative at 4°C. The interrupted sections were mounted on albuminised slides, dried and stained, H & E. These were examined for vasculitis. Sections, preceding and succeeding any vasculitis containing section, were rinsed in neutral distilled water. One section from each set was used for acid-phosphatase, one for alkaline-phosphatase, and one for β -glucuronidase. In the cases where all H & E stained sections were positive for vasculitis, some sections were incubated as negative control for each technique. Sections of liver were used both as positive and negative controls as indicated below.

(2) For the aryl-sulphatase technique a similar procedure was applied with the following variations.

- (a) Thickness of the sections was 15 μ .
- (b) Number of sections was 17.
- (c) Sections 1, 5, 9, 13 and 17 were stained H & E.
- (d) Intermediate sections were preserved at 4°C in neutral formal saline pending use.
- (e) Sections following H & E stained vasculitis positive sections were rinsed in graded saline, i.e. 0.85-2% instead of distilled water.

c. - Staining - The following methods were used to detect lysosomal enzymes. Incubation for all methods was carried out at 37°C.

- (1) Gomori's lead nitrate method for acid phosphatase activity (Pearse, 1968).

The incubation time was 35 minutes and the sections were not counterstained. For negative control, sections were incubated in a medium devoid of β -glycerophosphate.

- (2) Gomori's calcium phosphate method for alkaline phosphatase (Drury et al, 1967).

Incubation time was 90 minutes. Sections were counterstained for one minute with 1% aqueous eosin. A medium from which sodium β -glycerophosphate was omitted was used as negative control for the incubation of sections.

- (3) Hayashi et al's Naphthol AS-BI Glucoronide method for β -glucoronidase (GUR) (Bancroft, 1967).

Incubation time was 40 minutes. Negative incubation medium was devoid of Naphthol AS-BI glucoronide.

- (4) Rutenburgh, Cohen and Seligman's post-coupling method for arylsulphatase (Thompson, 1966).

Incubation time was overnight.

Unwashed tissues were used for fixation and sectioning.

From negative control medium 6-Benzoyl-2-naphthyl-sulphate was omitted.

B. - RESULTS

1 - Animals - Of the 38 ewes ten did not produce live lambs. One infected ewe from each group was barren, and 8 ewes in group "B" aborted their /

their lambs. Thirty-three lambs were born to 28 ewes of both groups. Four of them either died or were killed before due time, leaving a total of 29 lambs. Fourteen of these, 4 controls and 10 infected, comprised group "A", and the rest i.e. fifteen, 7 controls and 8 infected, belonged to group "B".

The presence of vasculitis was assessed on the basis of the H & E stained interrupted serial sections of blocks used for both histo- and immuno- chemistry. Vasculitis was present in CNS tissues of 17/18 infected lambs (groups "A" and "B" added together).

The characteristics of the lesion were as described in chapters IV and V and the segmental involvement of the blood vessel was once more noted when some of the serial sections revealed the interruption of the lesion.

In 4 of the lambs, 3 group "B" and one group "A", the lesion was very severe i.e. involving the majority of the blood vessels with dense populations of infiltrating mononuclear cells. In the rest, vasculitis was confined to 1-2 blood vessels infiltrated lightly with mononuclear cells. The only negative lamb in terms of vasculitis was a female from group "A". The lesion was absent in all of the controls.

The data examined for sex predisposition and litter size bias and no effect was found i.e. 8/8 males against 9/10 females and 12/13 singletons against 5/5 twins.

2. - Immunology

a. Skin test = (1) - Immediate reaction and caliper readings. -

Immediately after injection of the inocula the site of inoculation showed a macula which was followed by a papule after about 15-20 minutes. The latter lasted for about $1\frac{1}{2}$ - 2 hours.

There /

There was slight increase (1 mm) in the caliper readings of the skin thickness in all of the injected sites which lasted for 72 hours. However, apart from the short and immediate reaction and a moderate fairly uniform increase in skin thickness no changes were observed.

(2) - Histology - Examination of histological preparations of skin from the 4 inoculation sites revealed either no reaction or moderate infiltration of mononuclear cells together with eosinophils. In most of the cases the infiltration was confined to the stratum papillare of the dermis and were mainly distributed along the venules. Occasionally the inflammatory cells were aggregated in clusters between the hair follicles and the dermal vasculature. In only two cases was a diffuse infiltration of cells observed. In descending order of abundance, the cells comprised lymphocytes, eosinophils and macrophages. However, the pattern of distribution and the class of infiltrated cells were independent of experimental group, presence or absence of vasculitis in CNS, and the type of inoculum used indicating a mild allergic reaction possibly due to the substances of injected brain inoculum.

Arteriolitis was observed in one moderately hairy female lamb of group "A" in the skin specimen sampled from the site of ether-treated normal brain inoculation.

b. In vivo macrophage adherence test = (1) - In lambs

A summary of the statistical results is given in Table 17. As the exudate from one infected lamb was contaminated with blood, the results of counts of this specimen was not incorporated in the statistical assessment.

Basically lambs produced very little exudate in response to i.p. inoculation of sterile liquid paraffin.

The only statistically significant finding was a decrease of 18.9% in the number of macrophages in sensitised animals ($P < 0.05$). There was /

was a marginal fall in the number of the total cells, neutrophils, polymorphs and eosinophils in the infected animals. On the other hand, there was a slight increase in the number of lymphocytes compared with controls.

However, statistically these differences were not significant.

Another significant point which emerged from the comparison of the results between the individuals of the infected group was that there seemed to be a correlation between macrophage / lymphocyte counts and the severity of the lesion. The more severe the lesion the lower the number of macrophages and higher the number of the lymphocytes. These values ranged between 2.7 - 4% for macrophages and 87.0 - 92.8% for lymphocytes. In the individuals in which the lesion was milder the values for macrophage and lymphocyte counts ranged between 5 - 10% and 66 - 87% respectively.

(2) - Guinea-pigs - A summary of the results is given in Table 18.

The results were more encouraging in guinea-pigs than in lambs. They produced more exudate and with one exception there was no overlap between the values for sensitised and unsensitised animals. However, the only statistically significant finding was 37.6% decrease in the number of the macrophages in sensitised animals ($P < 0.001$). There was a considerable decrease in the values obtained for the total cell counts in sensitised animals, which apparently seemed to be a reflection of the low values obtained for macrophages, but this was not statistically significant ($P > 0.05$).

c - Immunofluorescent staining

(1) Demonstration of Ag localisation on the affected blood vessel wall, = Both direct and indirect techniques of immunofluorescence, using conjugated and unconjugated HSABD and the latter layered with MFl1, demonstrated moderate degrees of fluorescence. This was present in the adventitia of vasculitis affected blood vessels (Figs. 52 and 53), and could not be detected in the same coat of unaffected blood vessels (Fig. 54).

Occasionally, in the lumen and on the endothelial layer of a few affected blood vessels some fluorescent cells and spots were observed. However, similar fluorescing spots were present in the same locations of some control blood vessels which invalidated the significance of this observation.

Both control and vasculitis-affected blood vessels showed auto-fluorescence of the internal elastic lamina. The media of neither control nor affected blood vessels revealed any fluorescence.

The fluorescence of the adventitia of the vasculitis affected blood vessels indicates the presence of Border disease antigen in this coat.

(2) Examination for fibrin deposition = Positive control cryostat sections of fibrinous pleural exudate stained with FITC conjugated RASFS showed bright fluorescence at the sites of fibrin deposition (Fig. 56) thus confirming the potency of the serum. However, when this method was applied to vasculitis containing cryostat sections, no fluorescence was observed on the blood vessel wall except the auto-fluorescence of the internal elastic lamina. This finding indicates the absence of fibrin in the walls of vasculitis affected blood vessels.

3 - Histochemistry = a - Arylsulphatase:

Vasculitis was present in the CNS blocks of 12 cases used for the arylsulphatase method. In 11 animals infiltrating cells were not stained indicating /

indicating the absence of neutrophils in the population. In the remaining lamb pinkish/purple staining cells ranging from $1/25 - 1/42$ cells per "cuff" were present indicating a moderate neutrophil reaction. No other part of the affected blood vessels either in infected or control animals showed reaction. This lamb was a clinical shaker which in the last week of its life became stiff and recumbent and was found at autopsy to be affected by muscular dystrophy of the heart and thigh muscles. In addition thymus was small compared with other lambs of the same age.

b - Acid-phosphatase, alkaline-phosphatase and β -Glucuronidase

Vasculitis was present in CNS blocks of 13/18 infected animals from which the tissues were sampled for these techniques.

There were acid-phosphatase positive granules in the cytoplasm of the majority of the infiltrating cells. In most of them the reaction was weak i.e. the granules were few (2-3) and small. These cells were considered to be lymphocytes. However, 10-15% of the cells contained large and more numerous granules in their cytoplasm. These cells were considered to be macrophages (Fig. 55).

Infiltrating cells in vasculitis containing sections stained for alkaline-phosphatase were negative confirming the absence of neutrophils from the population of the infiltrated cells. The endothelial layer of the blood vessels and very minute areas of media were stained positively in two cases, which in view of the results of chapters IV and V are suggestive of minor degenerative and necrotic changes.

The β -Glucuronidase technique was capricious. However, in those preparations which were satisfactory some weakly staining cells were found which on the basis of their negative reactions for aryl-sulphatase and alkaline-phosphatase were assumed to be lymphocytes.

c. /

C - DISCUSSION

A total of 29 lambs born to ewes of separate groups inoculated with Border disease agent at 90 and 110 days of gestation were used for the production of vasculitis. The lesion was successfully produced in 17/18 infected lambs.

The presence of vasculitis in 9/10 Dorset-Horn x Cheviot lambs of group "A" infected at 90 days of gestation contrasts sharply with the findings in a similar experiment described in chapter V, where pure Dorset-Horn lambs were used but accords with the findings of chapter IV using same breed of sheep as the present experiments. This supports the hypothesis, put forward in chapter V, that there may be breed differences in response. Possibly the development of immune mechanisms takes place later in Dorset-Horn than in Cheviot x Dorset-Horn lambs.

Results of i.d. skin tests in the control and Border disease infected lambs do not confirm the hypothesis that a delayed type immune response is implicated in the vasculitis of Border disease. On the other hand, it may be that ether or heat treatment of Border disease inocula diminish the antigenicity of the Border disease agent, but possibly not that of the brain tissue itself. This attractive hypothesis could be supported by Gardiner et al's (1972) findings that no placental lesions resulted from the inoculations of ether- and heat-treated Border disease agent. The presence of the eosinophils in the dermis at inoculation sites could support the second part of this thought. The only question to be discussed in this regard is the specific fluorescence in the acetone fixed but ether treated sections stained with conjugated anti-Border disease serum. There are three possibilities for this:

- 1 - possibly once the agent is fixed with acetone does not lose its antigenicity.
- 2 - fifteen minutes, compared to overnight, treatment have not been enough to diminish the antigenicity.
- 3 - perhaps optimum concentration and dose were not used to evoke the skin reactions.

It would be interesting to find out the consequences of skin tests using untreated Border disease inocula, which requires further experiments outwith the concept of this work.

As i.d. inoculation of inactivated inocula apparently had no effect on the development of the vasculitis in CNS it seems that the arteriolitis observed in the skin of one case had been present before the application of skin test.

In vivo adherence of free floating macrophages to the peritoneal walls or viscera of the hypersensitive animals in response to the challenge of antigen have been shown by several investigators. Nelson and Boyden (1963) demonstrated this effect in response to s.c., i.v. and i.p. injection of tuberculin in hypersensitive guinea pigs with delayed-type hypersensitivity but failed to produce a similar response in guinea pigs with Arthus hypersensitivity. Hence, they concluded that the loss of macrophages from the peritoneal exudates due to the challenge of the antigen is a consequence of an immunological reaction and is a manifestation of a state of delayed type hypersensitivity. Corresponding results were obtained by other workers using BCG strain of Myco. tuberculosis (Nelson and North, 1965) and Gram-negative bacilli (Fauve, 1968) and finally the recognition of the reaction appeared in the WHO (1973) technical report series No. 519.

Results /

Results of this work, both in guinea-pigs and lambs, agree with the findings of the above mentioned workers, though in lambs very little exudate was produced hence smaller values were obtained. Findings of these tests support the hypothesis of delayed immunity in Border disease induced vasculitis cases. Therefore, it is suggested that the fall in the number of macrophages in the peritoneal exudates of both Border disease infected lambs and sensitised guinea-pigs is one of the manifestations of delayed type hypersensitivity. Furthermore, it provides encouragement for the further use of guinea-pigs in Border disease experiments which could facilitate the course of studies in this field. There appears to be no report, hitherto, concerning the use of *in vivo* macrophage adherence reaction in infections in which a viral aetiology is suspected, particularly in sheep. The results of this work may possibly lead to more widespread use of the test.

Direct and indirect immunofluorescent staining of Border disease induced vasculitis containing sections revealed fluorescence of the adventitia in affected blood vessels (Figs. 52 and 53) which was absent in control ones (Fig. 54). Thus localisation of Border disease antigen on the arterioles and small distributing arteries is demonstrated, and provides further evidence that the vasculitis is an immunological response.

The observed fluorescence was milder than that seen in the infected foetal lamb kidney cells stained with the conjugate HSABD. Considering the degree of the fluorescence it might be thought that:

1 - as in delayed type hypersensitivities, cell-bound antibodies are carried by cells and directed to react with the localised antigen/s leaving insufficient antigen to combine fully with the applied serum (antibody).

2 - the cell-bound antibodies differ from humoral ones and react less strongly with the antigen.

3 - The concentration of the antigen in foetal kidney cells fluorescing brilliantly with the conjugated HSABD is higher, possibly distributed more evenly, and has not been subjected to other antibodies and trauma of microtomy.

4 - The prepared HSABD is reacting weakly with the actual Border disease agent.

5 - Finally the agent producing vasculitis is different from Border disease inducing agent proper. This seems a very unlikely possibility in view of the co-existence of vasculitis and clinico-pathological Border disease in this experiment and the absence of vasculitis in many experiments using the same infective pool as inoculum but differing in other respects.

Lysosomal enzymes of inflammatory cells have been demonstrated by different workers such as Kaplow (1955), Straus (1958), Austin and Bischel (1961), Barka and Anderson (1962), Lorbacher et al (1967) and Hayashi (1967). According to their work neutrophils have a marked positive reaction for alkaline phosphatases and sulphatases but react weakly for acid phosphatases and β -Glucoronidase. Macrophages show marked to strong reaction for acid-phosphatase and β -glucoronidase, weak reaction for sulphatases and no reaction at all for alkaline phosphatases. On the other hand, lymphocytes show little acid phosphatase activity, no alkaline phosphatase or sulphatase activity and a variable β -glucoronidase activity.

Though the β -glucoronidase reaction did not work properly, the findings of this work suggest that the majority of the infiltrated cells are lymphocytes with 10-15% of them being macrophages (Fig. 59). Neutrophils are typically absent. Thus the histochemical findings are in accordance with light- and electron-microscopic observations.

The absence of fibrin deposition on the affected blood vessel wall was demonstrated by immunofluorescent technique. Thus, using the results of conventional /

conventional and immunofluorescent stainings for fibrin, it can be concluded with certainty that there is no fibrin deposition on the wall of the blood vessels showing vasculitis due to the Border disease agent.

D - CONCLUSION

Several cases of periarteritis were produced in lambs by the inoculation of pregnant ewes at 90 and 110 days of gestation. Localisation of the antigen on the blood vessel wall was demonstrated by immunofluorescent techniques using anti Border disease hyperimmune serum. An in vivo macrophage disappearance test implicated delayed hypersensitivity in the causation of the vasculitis. Application of the macrophage disappearance test to guinea pigs demonstrated the induction of delayed type hypersensitivity against the Border disease antigen. Infiltrated cells were demonstrated to be lymphocytes and macrophages. The absence of the fibrin deposition on the affected blood vessel wall is confirmed by immunofluorescent technique.

CHAPTER VII

THE MATERNAL LESIONS OF VASCULITIS

| | <u>CONTENTS</u> | Page |
|---|-----------------|------|
| INTRODUCTION | | 108 |
| A - MATERIALS AND METHODS | | 108 |
| B - RESULTS | | 110 |
| 1. Description of the lesion .. | | 110 |
| 2. Conditions under which the lesion was observed | | 110 |
| C - DISCUSSION | | 111 |
| D - CONCLUSION | | 113 |

CHAPTER VII

THE MATERNAL LESIONS OF VASCULITIS

INTRODUCTION

Since Border disease agent interacts with two hosts, i.e. the ewe and the foetus, it seemed important to carry out a systematic examination of the maternal tissues in respect of vasculitis. This part of the work deals with the light microscope, histological study of the tissues from the ewes and a few goats either used in the experiments of foregoing chapters or tissues available on file.

A - MATERIALS AND METHODS

Seventy-seven Cheviot and three Dorset Horn pregnant ewes from the experiments described in chapters IV and V comprised the animals for this study. Conception dates were determined as described in chapters IV and V. In addition, two goats inoculated at 54 days of gestation were incorporated.

Inocula, dose, dilution, routes and times of inoculations were as for groups "A" and "B" of chapter IV and group "A" of chapter V.

The techniques of post-mortem examination, sampling and the class of sampled tissues were as described for the female lambs in chapter IV, and, in addition to these, a set of at least 5 cotyledons was sampled from gravid uterine horn/s.

Fixation, preservation, processing and sectioning were identical to those described in chapters III, IV and V.

The staining techniques were H & E, von Kossa's method, Carbol chromotrope, Barlow's tribasic stain for the granules of mast cells, MGP + orange G and MSB as described in chapters III, IV and V.

Morphologic study of the lesion was from serial sections of paraffin embedded tissues. The drawings and the montage were carried out as in chapter /

chapter V. This involved serial sections of lesions of vasculitis in the placentome of two animals.

Also used were the filed paraffin embedded H & E stained sections from 80 ewes and 2 infected goats, arising from experiments conducted for the study of Border disease prior to 1971.

A summary of the number of ewes and goats in relation to their gestation at inoculation and the incidence of vasculitis is given in table 19.

B - RESULTS

1. Description of the lesion:

A vasculitis was observed which was similar to that described for the progeny (Fig. 57). However, in one instance, the infiltration by mononuclear cells was uniquely intense (Fig. 59) and in another the lesion was complicated by medial haemorrhage (Fig. 58).

Affected blood vessels were mainly located in the maternal side of the placentome and were confined to the basal muscular lamina. Where the foeto-maternal junction was intact extension of vasculitis from the maternal toward foetal side was evident, especially in longitudinally cut arterioles. The vasculitis became less marked as the maternal vessels narrowed at the maternal/foetal junction. The infiltrations were of the mononuclear cells.

The affected blood vessels were of the same class as described for lambs (chapter V) with a luminal diameter ranging between 15-50 μ , wall thickness of 5-20 μ and a $\frac{W}{L}$ ratio of 1:1.8-3.

Morphologic study of the paraffin embedded serial sections revealed a similar pattern of segmental distribution of the lesion on the affected blood vessels as in lambs and is demonstrated in Fig. 51b.

2. Conditions under which the lesion was observed:

The vasculitis was present in 21/120 infected ewes and 2/4 infected goats. In both species it was confined to dams inoculated at gestational ages between 30-70 days (table 19). Thus, considering only infected animals in this range, vasculitis was present in 21/105 infected ewes and 2/4 goats. None of the controls showed the lesion.

Vasculitis was not found in any tissues other than the placentome.

Vasculitis was only found between 17-75 days post-inoculation but no point of particular importance emerged from examination of the data (table 20).

Examination /

Examination of the data for the effect of route of inoculation also failed to reveal any trends.

In the other organs; namely skin, lymph nodes (prescapular and mediastinal), lung, heart, spleen, liver, gall-bladder, duodenum and kidney; other types of vasculitis were observed. These were mainly located adjacent to an acute or chronic bacterial, fungal- or parasitic-inflammatory focus. The infiltration of inflammatory cells^{in the majority of cases} was confined to the peri-arterial tissue but at times had spread to the other layers. The majority of the infiltrated cells in these types of vasculitis were either eosinophils or polymorphs (Fig. 60) which outnumbered mononuclear cells. Sometimes, invasion of veins and venules by these cells was also observed.

In the CNS vascular changes consisted mainly of venous cuffings.

In summary the maternal vascular lesions differed from the characteristic vasculitis, observed in foetal tissues and in caruncle, in following ways:

- a. the involved blood vessels were lying next to an acute or chronic infectious focus (particularly in lungs, lymph nodes and skin).
- b. the predominant cells were not mononuclear cells, but eosinophils or neutrophils.
- c. the blood vessels involved were not^{predominantly} arterioles or arteries.
- d. the infiltrated cells had spread more evenly without having been localised on the adventitia. Occasionally the infiltration was patchy.

C - DISCUSSION

Thirty-two different classes of tissues were examined from 160 ewes and 4 goats. Comparable vasculitis to that seen in lambs was observed only /

only in the caruncles or uterus. Apart from those described by Barlow (1972b), no other pathological changes attributable to Border disease was observed. The presence of vasculitis in the placentae of infected ewes and goats and its absence in controls, suggests that this lesion is one of the manifestations of the Border disease agent per se, though transient and inconstant.

Considering the cellular components of the infiltrate and the earliest post-inoculation time at which the lesion was observed, it seems plausible to suggest that the vasculitis in the caruncle is also an immunological response.

A wide range of gestational ages at inoculation, i.e. 30-130 days, was covered in this study. The lesion was confined to the inoculations carried out before 70 days gestation. The same range of inoculation times were examined in the study of vasculitis in progeny where in contrast it was found that the lesion does not occur in relation to injections earlier than 82 days gestation. Furthermore, in chapter III a reference was made to Silverstein's (1963a) work that the immunological competence in foetal lamb starts at around 60 days but responds at different times, to different antigens. Considering gestational stages at which the lesion occurs in ewes and progeny respectively, and the post-inoculation time at which the lesion is first seen, and finally Silverstein's findings it seems that there is a link between the sequences of events governing the occurrence of vasculitis in ewes and progeny. At the time of inoculation foetal immune mechanisms may or may not be developed. If they are, antigen from the mother reaches the foetus through the circulation and confronts the foetal immunological defences. This confrontation gives rise to the vasculitis in the progeny. But, if this system is not developed, the agent recirculates back to the mother in high titre and meeting the maternal immune mechanisms induces /

induces vasculitis in the placenta.

It is difficult to explain why the lesion does not spread to the other organs. It may be that because the foetus is non-immune the agent multiplies and reaches the "immune" maternal side in high concentration. Hence one has a situation of antibody co-existing with a vast excess of antigen (maternal antibody against Border disease develops 10 days p.i.). This situation would only persist locally as high titre antigen getting into the maternal circulation would quickly stimulate further antibody production and terminate the viraemia. Alternatively it might be that for the invasion of the agent to other organs ^{the} right milieu is not available and the confinement of the lesion to the placenta depends to the organotropism of the agent. However, a further study in depth of this aspect of the disease is outwith the scope of this work.

In animals vasculitis is recorded as a secondary event to a variety of causes, namely, to verminous, viral, bacterial and mycotic agents (Jubb and Kennedy, 1970). Extension of the inflammation from the tissues to the adjacent arterial and venular vessels is reported most often in the lung, lymph nodes, liver and digestive tract. It is suggested that the vasculitis in this chapter in organs other than the placentome have features compatible with this explanation and are not attributable to Border disease.

D - CONCLUSION

The examination of a wide range of tissues in ewes and a few goats revealed that the vasculitis in this class of animals is confined to the placenta and no other lesion attributable to Border disease is present in tissues other than placenta. The lesion is suggested to be immunological in origin.

CHAPTER VIII

GENERAL DISCUSSIONCONTENTS

| | <u>Page</u> |
|---|-------------|
| Introduction | 115 |
| A - Role of Border disease agent and the importance of age at inoculation | 115 |
| B - Post inoculation time at sampling | 117 |
| C - Relationship between the morphological findings and the type of immunological response | 117 |
| D - Comparison of the Border disease associated vasculitis with Periarthritis Nodosa (PAN) and associated conditions . | 121 |

CHAPTER VIII

GENERAL DISCUSSION

Introduction - To some extent the results and conclusions set out at the end of each chapter of this work prompted the experiments detailed in the succeeding chapters. The aim of this final chapter is to correlate the results of all the chapters and formulate a working hypothesis to explain the pathogenesis of the vasculitis in Border disease.

A - Role of the Border disease agent and the importance of age at inoculation

The presence of the vasculitis in the Border disease infected animals and its absence in the controls established the fact that this lesion is a manifestation of the Border disease agent. However, since not every infected animal had the lesion it is unlikely that vasculitis is the result of a simple direct attack of the agent on the vessel wall. In infected animals the vasculitis was present only when the inoculation was made at certain gestational ages and was not present in offspring infected at gestational ages earlier than 82 days. This finding was extended and confirmed through the experiments of chapter IV and the information gained was used for the experimental production of vasculitis, in a greater proportion of cases, during the experiments of chapters V and VI.

Examination of tissues sampled from ewes inoculated at different gestational ages revealed a similar transient and inconstant lesion in the caruncle of ewes inoculated when less than 70 days pregnant. Thus it appears that there is a limitation for development of the vasculitis in the two hosts, dam and progeny, which operates in opposite directions. In the former it was not found in ewes inoculated later than 70 days gestation and required a survival time of at least 17 days whilst in the latter the lesion only occurred following injection at > 82 days of gestation.

This age relationship of the lesion suggested that the vasculitis could be an immunological phenomenon. Having said this and considering the presence of persistent /

persistent infection in Border disease (Shaw et al, 1967; Barlow and Gardiner, 1969) one point seems to need explanation. Why does the vasculitis not occur in the offspring of the ewes inoculated earlier than 82 days? Changes from one pathological pattern to another in different ages of animals have been shown in viral infections such as Blue Tongue (BT) of sheep (Richards and Cordy, 1967) and Lymphocytic Choriomeningitis of Mice (LCM) (Traub, 1935; Volkert and Hannover Larsen, 1965). Richards and Cordy showed that the character of the CNS lesions in sheep and mice due to BT virus vary with age of the host. Their findings indicated that the young foetal sheep responds to BT virus by developing cerebral anomalies, whereas older animals respond with a non-suppurative meningo-encephalitis. The time during which the characteristics of the lesion change from one type to the other was deduced to be between 70 - 90 days of foetal life. Traub (1935) obtained similar results with LCM virus. Later, Burnet and Fenner (1949) recognised the importance of the persistence of LCM virus in the mouse even when no lesions are present. Together with the erythrocyte chimerism in twin cattle (Owen, 1945), the persistent infection of the mouse with LCM virus formed the basis of the concept of "self recognition" which later was to develop into one of the most interesting aspects of modern immunology, i.e. immunological tolerance (Billingham, Brent and Medawar, 1953).

The absence of vasculitis in the progeny of ewes infected earlier than 82 days gestation could thus be explained on the basis that the offspring of earlier infections recognise the Border disease agent as "self" and develop an immunological tolerance to the agent in respect of vasculitis.

However, in ewes the condition seems to be different. As at the time of inoculation the immune mechanisms of the foetus are not developed, if the agent reaches developing CNS tissue of the foetus, through maternal circulation and placenta it could replicate and recirculate to the dam. Hence the agent would confront the now activated immune mechanisms of the mother. Such a confrontation of the high titre antigen with the maternal antibodies in excess of antigen is a tenable explanation for the vasculitis in the caruncle.

B - Post-inoculation time at sampling. Another factor relevant to vasculitis which emerges from the experiments of chapters III and IV and was supported and confirmed by the experiments of chapters V and VI was the importance of post-inoculation time at sampling. The earliest times ^{for fully developed lesion} were 21 days in progeny and 17 days in ewes respectively.

Interaction of these two factors, age at inoculation and time to the first appearance of the lesion accords with the immunological hypothesis put forward in this work and immediately raises the question as to the type of immunological responses involved. This question may be partially answered here though other part/s will be discussed in later paragraphs.

Hitherto, attempts to demonstrate antibodies produced by the foetal lamb against Border disease have been unsuccessful except in one experiment (Gardiner, et al, 1967). Even then this investigator in his following experiments (Gardiner, 1973) concluded that the 7S γ -globulin demonstrated in the sera of Border disease affected foetuses / lambs are not specifically anti-Border disease antibodies. Thus it seems that humoral antibodies are not involved in the development of the vascular lesion in the progeny, and thus the lesion is not a product of type I (anaphylactic) or type II (cytotoxic) hypersensitivity reaction of Coombs and Gell's (1968) classification. One is left with either type III (Arthus reaction, Serum sickness) or type IV (delayed type hypersensitivity, cell-mediated immunity) allergic reactions which are the subjects of discussion hereinafter.

However, one point seems worthy of notice. Gardiner's materials for examination were obtained from the progeny of the ewes inoculated with the Border disease agent at earlier than 82 days of pregnancy. Therefore, it would be quite important to demonstrate antibodies against Border disease in the sera of foetuses/lambs from the ewes infected at gestational ages later than this, but until this is done there is no reason to abandon the present hypotheses.

C - Relationship between the morphological findings and the type of immunological responses

The morphological study revealed that Border disease induced vasculitis is characterised /

characterised by a segmental infiltration of mononuclear cells, mainly in the adventitia, and a minor degeneration. Microscopically and histochemically, it was shown that the predominant constituent cells of the infiltrate were typical - and atypical - lymphocytes and macrophages. Plasma cells and neutrophils were absent. The absence of plasma cells in the infiltrate confirms the lack of either circulating or locally produced humoral antibodies in Border disease vasculitis, a finding which was shown by Doherty, Reid and Smith (1971) in Louping-ill induced vasculitis.

Predominance of lymphocytes and macrophages in the lesion raises questions regarding their origin, how they get to the site of the lesion and why do they infiltrate the adventitia of arterioles and small distributing arteries? The first two questions were answered through the light- and electron- microscope studies as it was shown that the lymphocytes originate from the circulation traversing the affected arteriolar or arterial walls to the adventitia. Failure to demonstrate migration of the macrophages by the same route does not deny the concept that they too might have originated from the blood monocytes. Recent works (Konigsmark and Sidman, 1963; Huntington and Terry, 1966; Stenwig, 1972; Adrian and Williams, 1973; and Kitamura, 1973) on the origin of the macrophages in the injured CNS suggests that blood monocytes are the source. The weak possibility of the transformation of the lymphocytes to macrophages (Nelson, 1969) is unlikely since electron-microscopically neither intermediate cells nor in situ mitosis were observed. Furthermore, cytokinetic studies do not support the view that mononuclear phagocytes are derived from lymphocytes (WHO, 1973). Thus, it is concluded that the infiltrated lymphocytes and macrophages both originated from the blood.

However, it is not known why these cells migrate to the site of the lesion, which infiltrate first, and what is their relationship to the type of the allergic reaction involved? A possible explanation is that once the Border disease antigen reaches the foetus whose immune mechanisms are either developing or /

or fully developed, and through circulation gets to the CNS, it replicates and localises in the adventitia of the arterioles and small distributing arteries. Meantime, somewhere outside the CNS, some lymphocytes contact the antigen. These become activated and produce antibodies on their cell surface against the Border disease antigen, circulate in the body chemotactically traversing the arterioles and small arteries reach the site of antigen localisation and there react specifically. Once in the adventitia they release different molecular mediators (Tompkins et al, 1970; Allison, 1972; WHO, 1972) such as macrophages chemotactic factor (MCF), macrophages inhibiting factor (MIF), macrophages disappearance factor, and skin reactive factor (SRF). MCF attracts the macrophages from the circulation to the site of injury and under the influence of MIF they become stabilised. Some mediators also activate macrophages which then show increased phagocytic capacity to take up effete products of the combat (secondary lysosomes) and meantime interact with viral antigen on the surface of the infected cells. In this way macrophages play a role in the host's defence against the infection while at the same time contributing to the production of the lesion by release of lysosomal enzymes. The development of secondary lysosomes and degenerative changes were observed histochemically and by light- and electron- microscopy. This reasoning may be further supported by the results of chapter VI in which both localisation of Border disease antigen was demonstrated in the adventitia of affected blood vessels and macrophage disappearance factor was shown. However, the investigations for the SRF were not fruitful for reasons the majority of which have already been discussed in chapter VI.

"In practice, however, it is exceedingly difficult to differentiate with absolute certainty a type IV from a type III reaction" (Coombs and Gell, 1968). "Absence of circulating antibodies may be a pointer and the delay in appearance of the test reaction is certainly a useful guide". Nevertheless, one must try to compare the vascular changes in type III hypersensitivity reactions (e.g. Arthus reaction and Serum sickness), Border disease induced vasculitis and type IV hypersensitivity reaction. The basis of type III reaction is the presence of circulating /

circulating antibody, but as was discussed earlier, no circulating antibodies have yet been demonstrated against Border disease antigen. Goddard (1958) studied the vascular changes of Arthus phenomenon in rabbits. In summary the essential findings were:

- a- Venules = Luminal ingrowths leading to valve-like structures.
- b- Capillaries = Compression of capillaries.
- c- Arterioles = Luminal narrowing and obstruction, detachment and loss of endothelial cells, medial thickening + overwhelming oedema + fibrin deposition + fibrinoid degeneration + dominance of polymorphs and eosinophils in the infiltrate + perivascular hystiocytic granulomata with occasional arterialisation of the granulation tissue.

Germuth (1953) studied the pathological changes involved in Serum sickness of rabbits. In addition to glomerulonephritis, lymphadenitis, granulomatous lesions with giant cells in spleen, he found cardiovascular lesions consisting of:

- a- Intimal changes of arterioles and aorta = Subendothelial leukocytic infiltration of arteries and aorta, oedema and infiltration by neutrophils and some mononuclear cells. With time the neutrophils were replaced by mononuclears and by day 15 fibroblasts had replaced the infiltrated cells. The repair of the vascular lesion without scar formation occurred by day 28 and eventually no lesion was present.
- b- Media and adventitia = In these coats the lesions were similar to those of intima, with the addition of medial necrosis and fibrinoid degeneration which sometimes extended to the adjacent connective tissue.

No similarity was found between Border disease induced vasculitis and the pathologic changes of Arthus type vasculitis. However, the Border disease induced vasculitis showed some similarities to the pathological changes in Serum /

Serum sickness. These were oedema and predominance of mononuclear cells in the inflammatory reaction. Even so, in the vasculitis of Border disease the oedema was very moderate, confined to peri-adventitial tissues of arterioles and was not present in every affected vessel. Moreover, the time at which mononuclear cells were chiefly observed was different from that in Serum sickness. In Serum sickness mononuclears appeared just before the healing stage, whereas in Border disease associated vasculitis these cells were present from the beginning of the lesion and no change of cell type occurred. Furthermore, in the time required for the development of Border disease associated vasculitis, Serum sickness vasculitis has developed, progressed and resolved. Thus in Serum sickness, tissues sampled at 21 - 28 days post-inoculation would show no vasculitis, whereas in Border disease tissues sampled even 6 months p.i. showed no evidence of healing. Thus it seems that the weight of evidence is in favour of type IV allergic reactions of Coombs and Gell's (1968) classification of hypersensitivity reactions. However, it must be remembered that cell mediated immunity^{of this type} is only an established fact in guinea pigs.

D- Comparison of the Border disease associated vasculitis with Periarthritis Nodosa (PAN) and associated conditions

The complete morphologic study showed the lesion to be microscopic nodular and segmental and mainly confined to the adventitia of the affected vessel. These features resemble periarthritis nodosa or necrotising angitides and persuade the worker that he should compare the Border disease associated vasculitis with those similar vasculitides in the literature.

Rokitanski in 1852 was first to mention periarthritis nodosa (polyarteritis nodosa, essential polyangitis, panarteritis nodosa) in his observation, but it was Kussmaul and Maier who in 1866 clearly defined the gross and microscopic changes of this condition and called it periarthritis nodosa (Bruetsch, 1971). Over a century has passed since the first report and yet the aetiology of the lesion is not fully understood. New conditions sharing one or more characteristics of the lesion are frequently added to the complicated literature of this phenomenon. /

phenomenon. Most scientists now consider all these angiotides as a large group "the Collagen Diseases", (Aita, 1963). Definite members of this group are PAN, systemic lupus erythematosus (SLE), giant cell arteritis, thrombotic thrombocytopenic purpura, dermatomyositis, scleroderma, and rheumatoid arteritis. However, other conditions such as hydralazine poisoning, allergic vasculitis and Serum sickness, granulomatous arteritis, Wegener's granulomatosis, aortic arch arteritis, necrotising arteritis of rheumatoid arthritis, polymyositis, some myopathies associated with carcinomas, rheumatic fever and spondylitis are considered to be possible members of collagen diseases. Recognition and diagnosis of each condition is based on the clinical and paraclinical findings, but on the basis of microscopic histopathologic findings they share a common feature i.e. necrosis of blood vessel wall. Therefore, Zeek (1952) in her valuable review classified different conditions of this nature under "Necrotising angiotides" which comprise hypersensitivity angiotitis, allergic granulomatous angiotitis, rheumatic arteritis, PAN, and temporal arteritis. Some of these conditions such as temporal arteritis, rheumatic arteritis and scleroderma either are not reported in animals or have not been studied in depth. However, there are conditions in veterinary medicine which do share some characteristics of pathological lesions of collagen diseases. Examples are the vascular lesions of "equine viral arteritis", "malignant catarrhal fever of cattle", "sporadic bovine encephalomyelitis", "PAN", which the latter is reported in cattle, dogs, pigs, horses, deer, sheep (Jubb and Kennedy, 1970) and cats (Campbell, Fox and Drake, 1972). Nevertheless, polyarteritis in sheep is very rare and the examination of the literature in this respect revealed only 3 sketchy reports (Helmboldt, Jungherr and Hwang, 1959; Martin, 1961; Stehbens, 1963).

Therefore comparison of the vasculitis observed in this work with other conditions showing similar angiotides in sheep is impractical. Hence, a comparison is made in tables 21 and 22 with the necrotising angiotides of Zeek's (1952) classification and similar conditions in different species which may display periarteritis.

Earlier /

Earlier in this discussion and in chapter V the possibility of some vasculitides such as hypertensive arteritis and arteriolitis, atherosclerosis, Arthus type vasculitis were ruled out. In terms of predilection site of the lesion, class and coat of the affected blood vessels the population of infiltrated cells, the type of the degeneration and stage of the lesion, it would appear that the dissimilarities are too great to consider Border disease associated vasculitis identical with any of above mentioned angitides. It would seem that the progress of the lesion is very slow since no evidence of healing of the lesion has been seen in this study which covered foetuses from 103 days to lambs of 180 days. Further studies aimed at establishing the duration of the lesion after its development are needed. It seems that the Border disease associated vasculitis is a unique and separate entity.

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